# Identifying Gene Expression Biomarkers for Venetoclax and Bortezomib Resistance in Multiple Myeloma

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## ABSTRACT

Multiple Myeloma (MM) is the second most common hematologic malignancy. Despite the advancements in treatment approaches in the last decade, the prevalence of refractory disease leading to relapsed cases has been a major challenge. A wide range of intricate genetic heterogeneity demonstrated by myeloma patients is a credible explanation for the diverse treatment responses observed in patients sharing the same treatment regimens. Pertaining to this, the study aims to identify predictive gene expression biomarkers that forecast response to BCL2 inhibitor venetoclax and treatment outcome to proteasome inhibitor bortezomib. In this study, samples from MM patients were characterized into sensitive and resistant, (1) based on ex vivo response to venetoclax treatment (Resistant n=21; Sensitive n=21), and (2) based on their bortezomib treatment outcome in clinical profiles (Resistant n=12; Sensitive n=15). Associations between the different gene expressions and drug responses were studied using statistical and bioinformatic tools. As a result, we identified that significant (p-value <0.05) overexpression of 36 genes and downregulation of 38 genes appeared to confer resistance to venetoclax drug response in MM patients. Additionally, the functional association of these genes with pathways was determined using a pathway enrichment tool. Furthermore, the study provided evidence that cytogenetic alterations t(11;14) and t(4;14) are significantly (p-value <0.05) associated with differing venetoclax response in MM patients. These findings demonstrated that gene expression biomarkers and chromosomal translocations play a significant role in regulating venetoclax drug response in MM, which can be further utilized to personalize treatments for patients. The knowledge obtained from this work best applies in personalized medicine; whereby fitting treatments to an individual patient's genomic landscape will enhance patient outcome.



***Keywords***: *Multiple Myeloma, Biomarkers, Venetoclax, Bortezomib, Bioinformatics, Personalized Medicine*

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AID | Activation Induced Deaminase |
| AML | Acute Myeloid Leukemia |
| BCR | B-cell Receptors |
| BM | Bone Marrow |
| BMSC | Bone Marrow Stromal Cell |
| Bz | Bortezomib |
| CLL | Chronic Lymphocytic Leukemia |
| CNV | Copy Number Variations |
| CPM | Count Per Million |
| CSR | Class Switch Recombination |
| DEGs | Differentially Expressed Genes |
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribonucleic acid |
| DSB | Double Stranded Breaks |
| ECM | Extra Cellular Matrix |
| EMA | European Medicines Agency |
| FDA | Food and Drug Administration |
| FE | Fisher's Exact |
| FISH | Fluorescence In-situ Hybridization |
| GC | Germinal Center |
| HDAC | Histone Deacetylase |
| HSC | Hematopoietic Stem Cell |
| Ig | Immunoglobulins |
| IMWG | International Myeloma Working Group |

| | |
|---|---|
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| LDH | Lactose Dehydrogenase |
| LOH | Loss of Heterozygosity |
| MGUS | Monoclonal Gammopathy of Undetermined Significance |
| MM | Multiple Myeloma |
| MRI | Magnetic Resonance Imaging |
| NDMM | Newly Diagnosed Multiple Myeloma |
| NGS | Next Generation Sequencing |
| ORR | Overall Response Rate |
| OS | Overall Survival |
| PCD | Plasma Cell Dyscrasia |
| PCL | Plasma Cell Leukemia |
| PET/CT | Positron Emission Tomography-Computed Tomography |
| PI | Proteasome Inhibitor |
| PM | Personalized Medicine |
| RBCs | Red Blood Cells |
| RNA | Ribonucleic acid |
| RRMM | Relapsed or Refractory Multiple Myeloma |
| SDSS | Selective Drug Sensitivity Score |
| SHM | Somatic Hypermutations |
| SMM | Smoldering Multiple Myeloma |
| VEGF | Vascular Endothelial Growth Factor |
| WBCs | White Blood Cells |
| WHO | World Health Organization |

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# 1. INTRODUCTION

According to the latest fact sheet by the World Health Organization (WHO), new cancer cases have risen to approximately 18.1 million and cancer has taken the lives of 9.6 million people in 2018. There has been a huge reduction in the overall mortality rate of cancer patients in the last 20 years [1]. This dramatic improvement can be attributed to new treatment strategies, especially immunotherapy and targeted treatment approaches. However, a major issue in cancer treatment (especially in hematologic malignancies) remains the emergence of refractory disease which ultimately leads to reduced life expectancies [2]. The higher number of relapsed cases caused due to drug resistance, impose a serious clinical and economic burden, globally. The fact that this burden remains while newer strategies for treatments are developing, points to a gap between the expanding knowledge from cancer research and the translational ability of this information in the clinical setting.

A series of complex mechanisms and increased disease heterogeneity of cancer makes it difficult for one-size-fits-all kind of treatment approach to work effectively. The innate variability of treatment responses in different patients paves the way for personalized medicine. It is a promising approach according to which medical treatments are tailored based on distinct patient characteristics. Personalized medicine can be applied best in the context of analyzing genetic variation in patients to predict better prognosis, diagnosis, or treatment for individual patients. However, the successful clinical application of this approach depends on the availability of pre-existing validated knowledge about markers that can indicate those genetic variations.

Thus, in this study, we attempt to identify predictive biomarkers that can indicate resistant or sensitive responses to venetoclax and bortezomib treatments in Multiple Myeloma (MM). With this approach, in future, individual MM patients can be stratified into different treatment subsets based on their predictive responses. These stratifications will help to minimize disease relapse. Therefore, it is increasingly imperative for current and upcoming MM research to investigate the role of genetic data in patient care, to bring about individually tailored treatment approaches. This has the potential to alleviate the relapse burden in MM and improve the overall patient outcome.

# 2. LITERATURE REVIEW

## 2.1 Overview of Hematologic Malignancies

Hematopoiesis is a tightly regulated process of blood cell formation and development in which multipotent hematopoietic stem cells (HSCs) differentiate into mature blood cells with specific fixed functions to perform in the human body [3]. The adult hematologic system is comprised of about 10 types of blood cells that can be broadly classified into leukocytes (White Blood Cells (WBCs)) that are responsible for the human immune response, erythrocytes (Red Blood Cells (RBCs)) that transport oxygen and carbon dioxide and thrombocytes (Platelets) that are responsible for blood clotting. The system that houses the process of hematopoiesis in adults includes bone marrow, liver, spleen, and other lymphatic tissues. Intricate gene expression coordination strictly regulated by multitude transcription factors is what shapes the fate of HSCs to produce mature progeny [4].

Hematologic malignancies are a broad range of neoplastic transformations that affect different blood cell types and at different levels of their development. Disruptions in the genetic regulation of hematopoiesis at different points cause different types of malignancies [4]. Altogether these malignancies can be broadly classified into lymphoid malignancies, effecting the cells of lymphoid lineage, and myeloid malignancies, occurring in cells of myeloid lineage [5]. According to a combination of lineage, morphology, immunophenotype, genetic and clinical features, the World Health Organization (WHO) presented an updated classification of hematologic neoplasms in 2008 that classifies them into around 60 subtypes [5,6] (*Figure 1*). Hematopoietic neoplasms account for being the sixth most common group of cancer malignancies in the world and are more often occurring in the male population compared to females [7]. There exists a wide range of etiological diversity between the different subtypes of hematologic neoplasms, thus there is a poor understanding of the causative factors contributing to hematologic malignancies [8]. In this study, we will be focusing on Plasma B Cell malignancy: Multiple Myeloma.

## 2.2 Plasma Cells

Plasma B cells originate in the secondary lymphoid organs (e.g. lymph nodes) from differentiated antigen-activated B cells and soon after their formation they travel and inhabit the bone marrow [9]. They are a major element of humoral immunity because of their ability

*Adapted from Severson, C. (2016)*

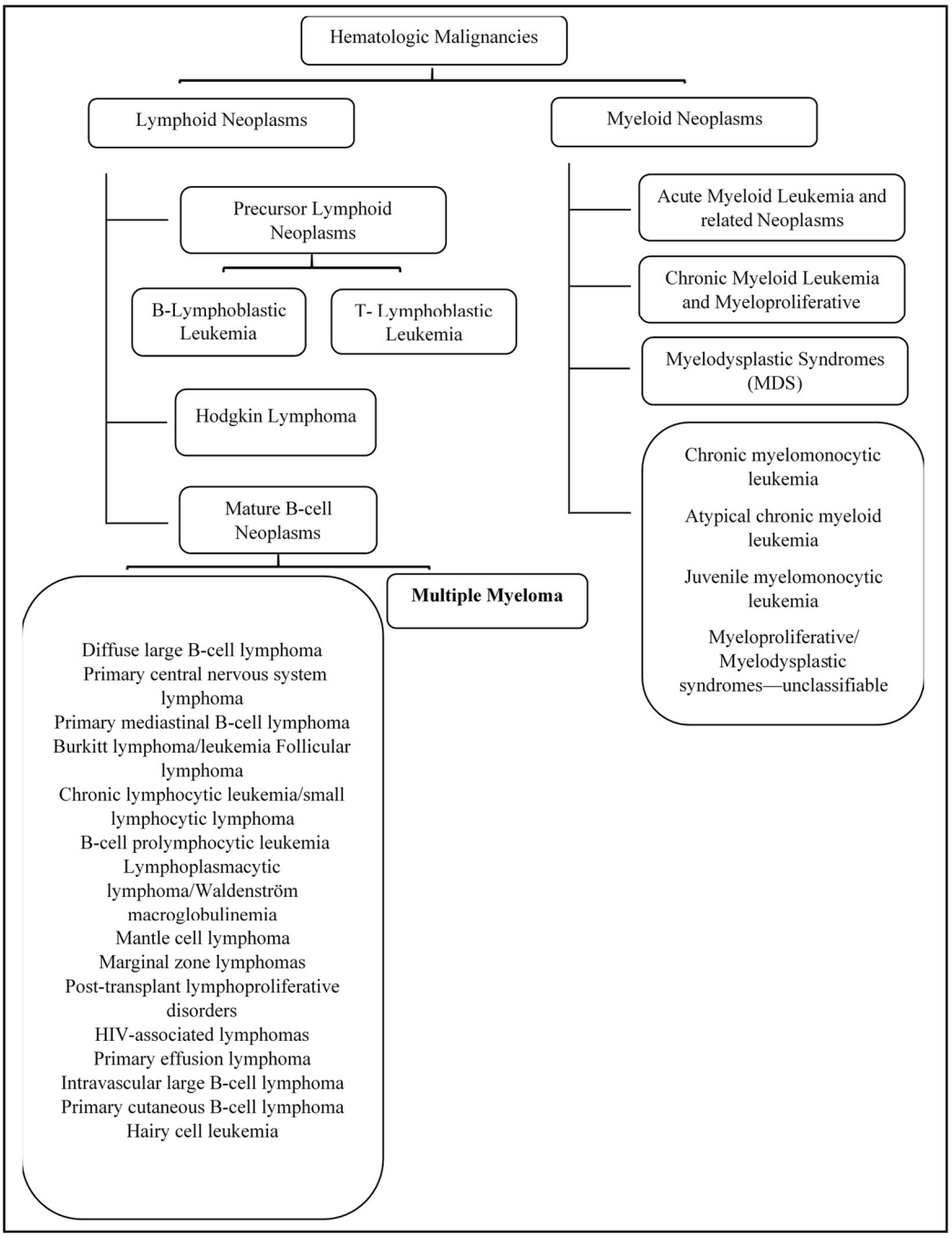


*Figure 1. Classification of Hematologic Neoplasms.*

to produce high quantities of antibodies and because of their extended life period [10]. Terminally differentiated plasma cells undergo loss of surface markers that are otherwise quite common in B cells, like CD19 and CD20, which makes them difficult to identify [11]. Mature plasma cells express high levels of CD-138 (syndecan-1) and CD38 [11], while CD54 and CD319 are good indicators of human plasma cells [12, 13].

### 2.2.1 Plasma Cell Development

When immature B cells encounter antigens in secondary lymphoid tissues, they can differentiate into memory B cells or plasma cells [9]. Interactions between antigen specific helper T cell and B cell cause the activation, proliferation, and differentiation of B cells [12]. In the secondary lymphoid organs, there are two possible fates for the activated B-cells, (1) extra-follicular activated B-cells differentiate into low-affinity antibody-secreting short term plasma cells that remain in the secondary lymphoid tissue (they have less significance in malignancies) (2) Germinal Center (GC) activated B-cells differentiate into long-lived plasma cells that are located in the bone marrow and secrete high affinity antibodies [14]. Activated B cells along with helper T cells and dendritic cells together form the germinal center (GC) in the secondary lymphoid tissues [15,12]. Following activation in the GC, B-cells undergo somatic hypermutations (SHM) in the genes that are responsible for the Variable region of the B cell receptors (BCR) [16]. These genes are responsible for the binding specificity of the BCR, thus SHMs causing accumulation of mutations in the Immunoglobulin Heavy Chain (IgH) improve the specificity of the antigen specific B cells. Another modification process called Class Switch Recombination (CSR) occurs in genes located upstream of IgH constant region [17]. Together these genetic processes (SHM and CSR) lead to affinity maturation and are initiated by an enzyme called cytidine deaminase [18] and cause B cells to differentiate into plasma cells. B cell clones that have undergone the process to achieve high antigen affinity and specificity are then selected by a process known as positive selection [19] and are retained in the GC. Altogether, the processes in the GC contribute to induce differentiation of B cells into mature plasma cells that secrete antibodies with high affinity and specificity for several foreign antigens. B cells exit the GC as mature plasma cells which then migrate to the bone marrow and survive there as long-lived plasma cells to provide for long term immunity [14,20].

### 2.2.2 Plasma Cell Dyscrasias

Plasma cell dyscrasias (PCDs) are a mixed group of malignancies that are characterized by the clonal expansion of bone marrow plasma cells that secrete monoclonal Ig (M-component) in blood serum or urine [21]. The most common PCD is Multiple Myeloma [22]. PCDs begin in the germinal center after affinity maturation of B-cells and establishment of a clonal population of plasma B cells [23] which when uncontrolled, cause symptoms like lytic bone lesions and ultimately develop into B-cells neoplasms. On one end of the PCD disease spectrum, there is Monoclonal Gammopathy of Undetermined Origin (MGUS) which is relatively asymptomatic and lacks the symptoms of serious PCDs [24]. On the other end, PCDs include intermediately severe condition like Smoldering Multiple Myeloma (SMM) and malignant dyscrasia like Solitary Plasmacytoma, Multiple Myeloma (MM) and Plasma Cell Leukemia (PCL) [23]. These different stages differ with respect to many components like the severity of symptoms, M serum protein levels, the extent of clonal expansion and concentration of Ig chains [24]. Regardless of the different parameters of PCD stages, life-endangering neoplastic complications can severely harm patient survival and require immediate treatment measures. Amyloidosis (misfolded protein disorder where proteins with beta-pleated sheets form fibril deposits outside the cells) [25], Crow-Fukase syndrome [26] (complex multitude symptoms due to multiple organ failure) and Cryoglubulinemia [27] (proteins precipitate at temperatures less than 37 degree Celsius) are few such complications. For this study, emphasis will be on Multiple Myeloma, a malignant and more symptomatic stage of PCD.

## 2.3 Multiple Myeloma

Multiple Myeloma (MM) is an incurable disease that first initiates in the bone marrow from the clonal expansion of mature antibody secreting plasma B cells [28]. In this section, we will discuss in detail about MM, its progression, treatment, and resistance, which will be the basis of this study.

### 2.3.1 Epidemiology and Etiology

Multiple Myeloma (MM) accounts for about 12-13% of all hematological neoplasms diagnosed every year and nearly 1% of all the diagnosed cancers [29]. Annually, MM occurs in about 6 individuals per 100 000 people worldwide [30]. It is rare in people below the age of 30 [31], with the median age at diagnosis to be 66-70 years and is about 40% more common in men than women [32]. Regarding ethnic distribution, there are half as many patients of European descent, compared to Afro-Caribbean, and there is a lower incidence in Asians [33].

With the introduction of new therapeutic strategies and advanced diagnostic techniques, the survival rate of MM has significantly improved. Recently, it has been reported that the five-year survival estimate for all ages has improved to 54% from 36% between 2001—2005 [34]. Average overall survival (OS) in patients less than 65 years has significantly increased due to new treatment approaches [35]. However, despite the prominent improvements over the past years, MM remains an incurable disease.

The etiology of MM is not very well understood. Increasing age, male sex, African ethnicity, and history of Monoclonal Gammopathy of Undetermined Significance (MGUS) have been documented as one of the apparent epidemiological risk factors of MM [36]. Data-driven findings show that MGUS or Smoldering Multiple Myeloma (SMM) precede MM onset. Every year almost 1% cases of MM have progressed from preexisting MGUS [37]. Several studies conducted earlier have shown that MGUS can develop as early as 15 years before MM development, remaining silent and asymptomatic for years [38].

Genetic history of MGUS, MM or other B-cell related malignancies in the family has been known to cause MM. According to some studies, there is almost a 2 to 3-fold risk of developing MM in immediate relatives of patients with MM [39]. Familial risk of MM has been associated with seven genetic loci: 2p, 3p, 3q, 6p, 7p, 17p and 22q [40]. Familial risk indicates a role of genetic factors being involved in contributing to the etiology of MM. Genes involved in B-cell proliferation and survival pathways and apoptotic pathways are important genetic risk factors [39]. Apart from these, occupational and environmental exposure are also known risk factors. Exposure to radiation causes DNA damage which can lead to Ig translocations and dysregulation aiding in MM progression [41]. In agriculture, exposure to insecticides and pesticides can interfere with

metabolic pathways that are essential for normal homeostasis [42]. Studies have reported a small positive association between farming and MM [39]. Obesity also presents as a flexible risk for both MM and MGUS [39]. Recently, two studies showed that obesity can be associated with a high risk of MM [43,44].

Myeloma is also not a single disease entity [45] and its etiology can be associated with a wide range of occupational, lifestyle and genetic components. Although many association studies have been conducted, it is highly probable that many genetic factors and their roles in MM remain unidentified [40], thus, this research field demands more study cohorts to discover further such associations in MM.

### 2.3.2 Clinical Presentation and Diagnosis

Multiple myeloma's clinical presentation can widely vary from asymptomatic (originating from MGUS), where the disease is discovered incidentally, to showing potentially fatal symptoms (advanced malignant MM). Usually, patients with MM show symptoms of prolonged pain in the bones (commonly ribs and back) and fatigue, sometimes fractures, intermittent infections, and radiculopathy [12]. Renal insufficiency is caused because of monoclonal light chain precipitation in kidneys, and with hypercalcemia [46] is present in about 15-30% of MM patients [47]. Some infrequent symptoms of MM include hyperviscosity syndrome, soft-tissue deposits, and blood loss, which present in around 10% of MM cases [48].

The diagnostic hallmarks of MM are according to the original CRAB criteria, this criterion summarizes the major clinical manifestations of MM – Calcium levels (C), renal failure (R), anemia (A), and bone lesions (B) [49]. In 2014, the International Myeloma Working Group (IMWG) proposed an updated diagnostic definition of MM because, before these criteria were updated, a clinical indication of serious multiple organ damage was needed to diagnose MM [50]. The old criterion would have prevented early myeloma diagnosis thus postponing therapy. The updated diagnostic standards include the additional identification of biomarkers associated with the development of CRAB symptoms in patients. Patients not showing CRAB symptoms and presenting with bone marrow plasma cells greater than 60%, a serum-free light chain ratio of 100 or more, or an MRI with more than one focal lesion are diagnosed with MM according

to the IMWG [12,50]. Differences in disease diagnostic criteria between MM, SMM and MGUS are described in detail in *Figure 2*.

*Reproduced from Majumder, M.M. (2018)*

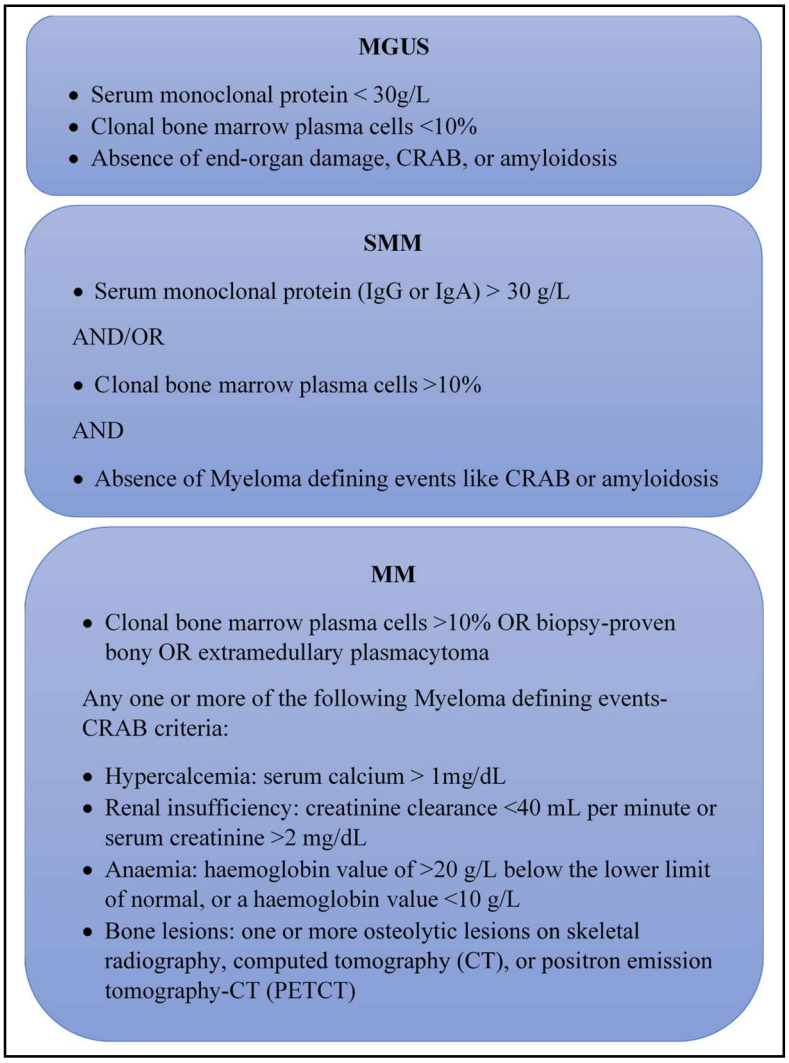


*Figure 2. Diagnostic criteria for MGUS, SMM and MM*

### 2.3.3 Multiple Myeloma Disease Progression

Multiple Myeloma originates from the neoplastic development of mature long-lived plasma B cells of the bone marrow. It is marked by genomic instability at many levels, including the deregulation of plasma cell development and incorporation of genetic alterations [51]. The events leading to malignant plasma cell population are known to be initiated as early as the B cell maturation stage [51]. During B cell maturation, B cells undergo affinity maturation with the help of two processes, somatic hypermutation and class-switch recombination as explained in *Section 2.2.1*. Activation-induced

Deaminase (AID) required for both SHM and CSR mechanisms results in Double Stranded Breaks (DSBs) in the immunoglobulin loci [52]. Most of these DSBs are repaired by the cellular environment, however, some persist and can lead to genomic rearrangements [53] to ultimately give rise to malignant plasma cell population. These genomic rearrangements cause many alterations such as chromosomal translocations, IgH translocation and are the main molecular hallmarks of MM, leading to the immortalization of myeloma initiating cells [54]. These initial genetic events can result in development of MGUS, which is a pre-malignant condition that is found to progress to MM in most cases [37]. Along with these primary events during B cell maturation, once the myeloma propagating cells reach the bone marrow, the microenvironment plays a very important role in supporting their growth and development [53, 55]. While the primary events like, translocations, hyperdiploidy and mistakes in DNA break repairs occur early and are responsible for the progression from healthy post-germinal center B cells to malignant plasma cell populations, the secondary genetic events including copy number variations, Loss of Heterozygosity (LOH), mutations acquired in the BM microenvironment and epigenetic factors, occur later and assist in the clonal evolution of MGUS to MM to Plasma Cell Leukemia (PCL) [12]. PCL is at the end of the disease spectrum when malignant plasma cells no longer need the BM microenvironment and can independently proliferate outside the BM in the blood stream [56]. *Figure 3* is a schematic representation of disease initiation to MM disease progression. The progression of the disease from premalignant conditions to the severe malignant disease involves complex clonal evolution, cumulative genetic abrasions, and epigenetic changes. As the disease progresses, there is a constant selection of the best clone to survive independently and the clones existing before them are replaced with more aggressive ones, thereby establishing a progressive disease and treatment resistance [57].

### 2.3.4 Genetic Environment and Clonal Evolution

Multiple Myeloma's genomic landscape is quite complex and incorporates many genetic aberrations. There are two major subtypes of MM, based on genetic alterations, (1) Hyperdiploid MM (H-MM) and (2) Non- Hyperdiploid MM (NH-MM) [58]. The H-MM variant is described by the multiple trisomies mainly effecting odd chromosomes 3, 5, 7, 9, 11, 15, 19 and 21 [59]. This is known to provide a favorable environment for

the expansion of malignant clones. On the other hand, NH-MM characteristically includes *IgH* translocations that are largely missing in H-MM [60]. While H-MM is

*Representation inspired from Bianchi, G., & Anderson, K.C. (2014)*

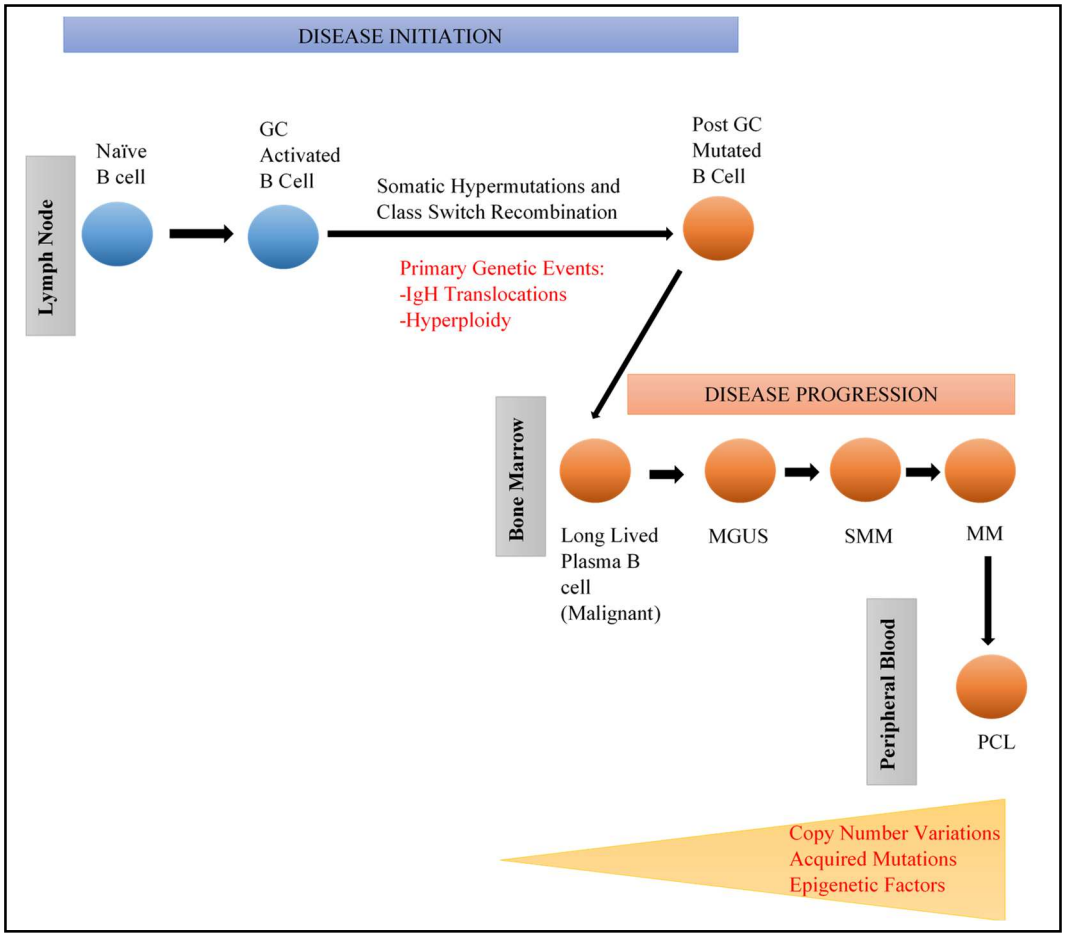


*Figure 3. MM disease progression from naïve B cell to malignant plasma cells to Plasma Cell Leukemia (PCL).*

known to occur early in plasma cell clonal expansion, NH-MM translocations are seminal incidents in disease progression [39]. However, both these genetic events are considered as early alterations in MGUS and are not known to independently carry the transformation into MM. Genetic aberrations in MM include somatic mutations, copy number variations (CNV), chromosomal translocations and mutations that alter major pathways in the BM microenvironment [53].

In the germinal center of the lymph node, during gene editing sometimes the DNA breaks are incorrectly joined which is when the NH-MM translocations take place [61]. These translocations result in the juxtaposition of the IgH with an oncogene [61]. This process locates the translocated product under a strong IgH enhancer leading to

overexpression of the oncogene [62]. The most commonly occurring translocations in multiple myeloma are t(11;14), t(14;16), t(4;14), t(14;20) and t(6;14) [63]. These translocations are generated by CSR and place several oncogenes like *CCND1, FGFR3* and *MMSET* in control of strong enhancers [64]. These are considered primary chromosomal events, while secondary events including deletion of 13q and 17p, as well as gain of 1q [65] are assumed to be acquired during the progression of MM and not initiating events.

As the disease progresses, there is an increase in the burden of mutation and genetic complexity, which drives the disease evolution [40]. In the past half-decade, due to cheaper and more feasible next generation sequencing technologies, vast numbers of recurrently mutated genes in multiple myeloma have been identified [12]. Almost about half of MM patients harbor mutations in genes from the Mitogen Activated Protein Kinase pathway (MAPK), that include driver genes like *BRAF, KRAS* and *NRAS* [66, 67]. Mutations in these genes, causes them to dysregulate cellular mechanisms like cellular proliferation, repair mechanisms, etc. A study conducted in approximately 450 myeloma patients further identified a long list of mutated genes including *FAM46C*, *DIS3, CYLD, TRAF3 and IFR4* [67]. Members of Wnt signaling pathways, adhesion factors like *VLA4*, cytokines (IL-6, BAFF, TNFα,) [68] and chemokine receptors like *CXCR4* are known factors in creating a suitable environment in the BM for malignant myeloma cells [69,70]. Various genes involved in the upregulation of NF-κB signaling play an important role in MM disease progression [71].

When compared to other hematologic malignancies, the mutation rate in MM is higher, however, it is lower than most of the solid tumors [72]. This favors the progression of malignant plasma cells and makes this disease even more aggressive. Additionally, MM is believed to follow Darwinian evolution led by competing clones [73], resulting in the best surviving clone to further expand. These sub-clones change in size and distribution across the course of disease based on which clone survives the best at which stage [73]. Studies conducted in MM patients demonstrate that the progression of sub-clones follows different patterns and can vary from individual to individual [73]. It has also been demonstrated that the number of clones changes with disease stage and treatments [74]. The complexity in the clonal evolution of MM makes it difficult for one treatment strategy to give favorable outcomes in all patients. This is the main motivation behind this project, as we attempt to identify differences in gene expression profiles and

cytogenetic alterations of MM patients resistant or sensitive to specific treatments.

### 2.3.5 Staging and Prognostic Factors

Altogether, three staging systems have been proposed for multiple myeloma so far: Durie Salmon System (DSS), International Staging System (ISS) and the Revised International Staging System (RISS) [75]. In the 1970s, the Durie Salmon system was implemented that used parameters like calcium, renal function, hemoglobin, and bone lesions to quantify the tumor burden in myeloma [76]. To develop more clinically sensitive yet simple staging criteria, the IMWG in 2003, proposed the ISS system that included PET/CT and MRI but excluded hemoglobin assessment [12]. The ISS system stratifies patients into three stages based on their serum albumin and serum β2-microglobulin [77]. It also predicted the overall survival in months corresponding to the three stages. A need for updated criteria arose because neither of the existing systems included chromosomal information in risk stratification. Therefore, in 2015, the ISS was revised to establish the RISS system [78] which classified based on existing ISS along with chromosomal aberrations. RISS stage I includes the patients in ISS stage I along with having chromosomal alterations not associated with high risk, like, 17p deletion or t(4;14) and having normal Lactate Dehydrogenase (LDH) levels. Stage III has patients with ISS stage III, high LDH and high risk associated chromosomal aberrations like t(11;14). Patients that fall neither in stage I nor stage III belong to stage II [79]. Additionally, these stratification systems also hold prognostic value for MM progression. Presence of del(17p), t(4;14) and t(14;16) detected by fluorescence in-situ hybridization (FISH) is associated with poor prognosis in patients and they are stratified as high-risk individuals [12]. *Table 1* shows a detailed classification of the three systems.

Prognostic factors related to MM progression were determined considering either the patient characteristics or tumor characteristics [75]. Patient-related prognostic factors shape the intensity of the treatment burden given to them while tumor-related factors indicate the tumor burden and disease progression [75]. Impaired kidney function indicated by serum creatinine level above 2mg/dL is a poor prognostic parameter concerning patient characteristics and elevated LDH is a poor prognostic factor indicating high disease burden [75].

*Adapted from Bianchi, G., & Anderson, K.C. (2014) and Majumder, M.M. (2018)*

| ***Stage*** | ***Durie-Salmon System (DSS)*** | ***International Staging System (ISS)*** | ***Revised International Staging System (RISS)*** |
|---|---|---|---|
| ***I*** | Hemoglobin >10 g/dL, serum calcium ≤12 mg/dL, absence of bone disease or solitary plasmacytoma, M protein <5 g/dL if IgG or <3 g/dL if IgA, and/or Bence-Jones proteinuria <4 g/24 h | Serum β2-microglobulin <3.5 mg/dL AND serum albumin ≥3.5 g/dL | ISS stage I and standard-risk chromosomal aberrations by iFISH and normal LDH |
| ***II*** | Meets criteria for neither stage I nor stage III | Meets criteria for neither stage I nor stage III | Not RISS stage I or III |
| ***III*** | One or more of the following must be present:<br><br>Hemoglobin <8.5 g/dL, serum calcium >12 mg/dL, extensive bone lesions, M protein >7 g/dL if IgG and >5 g/dL if IgA; and/or Bence-Jones proteinuria >12 g/24 h | Serum β2-microglobulin ≥5.5 mg/dL | ISS stage III and either high-risk chromosomal aberrations by iFISH or high LDH |

*Table 1. Risk stratification of MM based on three staging systems, Durie Salmon System (DSS), International Staging System (ISS) and Revised International Staging System (RISS).*

### 2.3.6 Treatment Terrain

Recent years have seen advancement in multiple myeloma therapy and even though it remains an incurable disease, the discovery of novel therapeutic strategies has improved the overall survival (OS) of patients [80]. Introduction of proteasome inhibitors like bortezomib and immunomodulatory drugs like lenalidomide have drastically changed the survival landscape of multiple myeloma [80]. Increased myeloma disease relapse led to the development of second-line proteasome inhibitors like carfilzomib that have proved to be highly beneficial for patients in the refractory phase [12]. Clinical trials have shown the effects of other therapies like histone deacetylase (HDAC) inhibitors, alkylating agents, and monoclonal antibodies, to be fruitful in improving patient outcome [81].

There has been a dramatic increase in the number of MM therapies, however, due to clonal heterogeneity observed in myeloma patients, sensitive cells may be eradicated but resistant subclones survive and thrive under suboptimal treatment [82]. Therefore, as much as determining the right treatment is important, deciding the synergistic drug combinations that can target even the resistant subclones so that relapsed cases can be reduced, is even more important. Further in this section, we discuss the mechanism of action of various treatments used in MM and detailed discussion of bortezomib and venetoclax which were used in this study.

***Proteasome Inhibitors (PIs)***: PIs are the backbone of multiple myeloma treatment, and are effective as both first line and refractory therapy [12]. Both newly diagnosed multiple myeloma (NDMM) and relapsed or refractory multiple myeloma (RRMM) patients have shown improved overall survival with PI treatment [83]. The main principle behind PI mechanism of action lies in increasing the misfolded/unchecked protein stress in the malignant cellular environment to dysregulate mechanisms so that cell is forced to undergo apoptosis. Proteasome degradation pathway is responsible for maintaining cellular homeostasis by degrading misfolded/unfolded proteins thereby regulating cellular proliferation, apoptosis, repair mechanisms, etc. [83] Three enzymes involved in polyubiquitination of the target protein, label it so that it can be detected and degraded by the proteasome. About 60% of cellular protein [84] is degraded by 26S proteasome consisting of 20S proteolytic unit and 19S regulatory unit. Once the ubiquitinated proteins are degraded by the 20S unit, cellular homeostasis is maintained. PIs inhibit this mechanism and increase cellular stress due to accumulation of proteins thereby inducing cell death [85]. It has been investigated that PIs are more effective on malignant cells as compared to normal cells because malignant cells have a high requirement for proteins to keep up with proliferation [83]. Bortezomib, carfilzomib and ixazomib are currently approved medications as PIs, whereas oprozomib and marizomib are still under clinical investigation for determining their efficacy and safety [12]. Use of PIs in combination with immunomodulatory drugs like lenalidomide enhances overall response rate (ORR) of patients [12].

***Immunomodulatory drugs (IMiDs)***: This class of drugs include lenalidomide, thalidomide and pomalidomide and have significantly improved the patient outcome in the clinical setting [86]. Like PIs, IMiDs have shown to be effective in both NDMM and RRMM patients including both transplant eligible and ineligible patients [12]. IMiDs are

known to have many actions including, anti-inflammatory, anti-angiogenic, cytotoxic and immunomodulatory, nevertheless, recently their role has also been demonstrated in ubiquitination for proteasomal degradation [87]. This action has been investigated and effects by targeting Cereblon (*CRBN*), which is a protein receptor for

E3 ubiquitin ligase complex. The ubiquitination of important transcription factors, IKZF3 and IKZF1, is commenced once IMiDs bind to *CRBN* thus, leading to their proteasomal degradation [88]. This degradation results in the reduction of *IRF4* and *cMYC* that are associated with MM disease progression [88]. However, for IMiDs to work through this mechanism, *CRBN* must be expressed in MM patients; downregulation of which could lead to a resistant mechanism to the drugs [89].

***Histone Deacetylase Inhibitors (HDACIs)***: Gene expression can be regulated without physically altering the genetic sequence, by post-translational modification in histone proteins [90]. Histones are proteins that help package large DNA into compact form in a cell. Histone tails can be modified by various processes like, acetylation, methylation, phosphorylation, etc. thereby leading to modulation of gene expression [91]. Histone lysine residue acetylation is regulated by two enzymes, one of them being Histone Deacetylase [91]. HDAC led deacetylation leads to a more compact conformation of DNA, thereby causing transcriptional repression [91]. Overexpression of HDAC causing hypoacetylation of histones is associated with poor prognosis not only in multiple myeloma, but many other cancers (e.g. colorectal, gastric, and pancreatic) [92]. Thus, HDACIs have great potential in cancer treatments. HDACIs initiate cell cycle arrest at G1 phase by upregulating *CDKN* inhibitor [91]. BCL2 family proteins which are proapoptotic are known to be upregulated when HDAC is inhibited resulting in apoptosis [93]. Studies have shown that HDACIs also interfere in stress responses and DNA damage responses by inducing double stranded breaks [91]. These drugs also inhibit heat shock proteins Hsp90 which is essential for the assemble of proteins involved in major cancer signaling pathways like Akt, ERK, Raf, etc [91]. Vorinostat is an HDACI that targets class I and class II HDACs, whereas panobinostat has shown about 10 times more inhibitory action against class I, II and IV HDACs [91]. Romidepsin, a class I specific HDACI, was approved by the FDA in 2006 and is effective for relapsed MM patients [91].

In this study, we investigate the response to two drugs, bortezomib and venetoclax. While bortezomib is a conventional treatment measure administered to almost every myeloma patient, venetoclax has not yet been approved for MM but has shown good potential in clinical trials. The following sections discuss them in detail.

**Bortezomib: Conventional Therapy**

In 2004, bortezomib (Bz) was the first in class boronic acid-based proteasome inhibitor approved by the FDA for first line of treatment in Multiple Myeloma. It is a reversible inhibitor of subunit β1 and β5 of proteasome 26S [83]. Quick start in treatment with Bz is beneficial for MM patients with renal insufficiency because modifying the dose is not required [94]. Bz has an inhibitory effect on NF-kB, which is overexpressed and helps in the proliferation of myeloma cells. It also inhibits the production of VEGF (Vascular Endothelial Growth Factor), which maintains the BM microenvironment by regulating angiogenesis [83]. Various mechanisms of actions against myeloma progression [95] make Bz one of the most potent anti-myeloma drugs in the landscape. However, bortezomib is usually given in combination with other drugs because of better outcomes. Studies show that patients with a combination of Bz and dexamethasone show improved responses without compromising with toxicities [96]. Bz has shown synergistic effects with melphalan and prednisone [97]. Frequent side effects include fever, peripheral neuropathy, infection, dizziness etc. Subcutaneous administration of Bz associates with the least adverse effects [98].

Bz shows great results in an initial response, but most patients relapse to Bz therapy. Many pathways of Bz resistance have been identified, like serine synthesis pathway and pentose phosphate pathway [99]. However, the mechanism of resistance can be more complex and varied based on an individual patient's genetic profile. Thus, for this study, we chose to investigate the response to bortezomib.

**Venetoclax: Emerging Therapy**

Venetoclax was been approved by the US Food and Drug Administration (FDA) for the treatment of in chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML). It is a selective BCL-2 inhibitor, a protein that is crucial for the regulation of apoptosis [100]. Apoptosis can happen via either the extrinsic death-receptor-mediator

pathway or the intrinsic mitochondrial pathway. BCL-2 anti-apoptotic family of proteins regulate apoptosis via the mitochondrial pathway, which gets activated in response to stress thereby resulting in loss of mitochondrial membrane potential. This releases cytochrome c into the cytosol, due to which the cell must undergo apoptosis [101]. Avoiding apoptosis by dysregulation of the BCL-2 family proteins is a well-known mechanism of disease progression in hematologic cancers [100]. This makes selective inhibition of BCL-2 an effective treatment strategy.

Venetoclax is a potent treatment approach with high efficacy and safety in CLL and AML patients [101]. Nonetheless, experiments conducted on multiple myeloma cell lines demonstrate that the positive effect of venetoclax even in the presence of 17p deletions (considered high-risk MM) [102]. 20% of MM patients present with overexpression of BCL-2,[103] suggesting that venetoclax could be an effective treatment. Although venetoclax is not yet approved for MM, studies investigating its effectiveness in MM are swiftly rising [101]. In this study, we chose to investigate *ex vivo* sensitivity of MM patient samples to venetoclax as a surrogate for response.

## 2.4 Drug Resistance in Multiple Myeloma

Relapse burden is high in MM due to the development of resistance against existing drugs. Concentrating on this challenging issue, many studies have been conducted to examine the causative factors for resistance against MM drugs (mainly chemotherapeutic agents). Overexpression of P-glycoprotein (*P-gp*) has been identified to interfere with multiple chemotherapeutic drugs by preventing the accumulation of drug molecules intracellularly, by this means causing resistance [104]. Dysregulation of genes involved in cell-cell interactions between Bone Marrow Stromal Cells (BMSCs) and MM cells can lead to adhesion of malignant cells to the BM stroma, thereby causing cell-adhesion mediated drug resistance (CAM-DR) [104]. Additionally, BMSCs play a role in overexpression of drug transporter genes like *ABCG2* which eventually contribute to CAM-DR. *HIF1A* and *LDHA* are actively involved in MM resistance [105]. A recent study discovered the association of upregulated *LDHA* and *HIF1A* with refractory MM in patients that were given Bortezomib and Melphalan therapy [105]. A member of the Ras family, *RASD1,* is known to play a significant role in mediating

resistance against dexamethasone in MM patients [104]. It has also been recognized that *CXCR4* is associated with bortezomib resistance in MM, through CAM-DR [104].

Many factors contribute to resistance against MM drugs, mainly genetic abnormities, epigenetic aberrations, and disturbed histone methylation [104]. All these affect genes and their regulation coupled with modifying various critical pathways. The most important take away from this section is that gene expression and regulation is central and therefore a precious tool that can be used towards making better treatment decisions, leading to better patient response. Additionally, mechanisms of resistance against therapeutics like PIs and immunomodulatory agents in MM are not very well understood yet [104], therefore requiring further focused research in this area. Thus, this study is addressing relevant challenges and findings from it could potentially pave the way for significant breakthroughs in MM disease management.

## 2.5 Personalized Medicine

In the early 1950s, observations of considerable variability in drug response among individuals initiated the discipline of pharmacogenetics [106]. It is a field that studies the genetic and epigenetic mechanisms underlying the variability in treatment responses. The commercialization of this field is now a modern and stimulating topic in healthcare, known as Personalized Medicine (PM) [106]. PM follows the objective that treatments are tailored in a manner such that they are as customized as the individual's disease itself. This revolutionary approach utilizes genomic and clinical information from patients to understand how a patient's unique profile makes them sensitive or resistant to medical treatment [107]. Due to its precise nature, PM not only has the potential to reduce disease relapse but also dampen financial and temporal burden. New drug discovery is a time and money consuming procedure, to which PM provides a great solution where existing drugs can be utilized to patients where they are most effective [107].

Valuable utilization of PM relies on the availability of rigid information about biomarkers [107]. Based on the use of biomarkers to predict a patient's response to treatment, patients can be stratified, and optimal therapeutic strategies can be selected. However, the full potential of PM has not been realized in the clinical setting due to the

poor understanding of clinically significant robust biomarkers [107]. This calls for more multidisciplinary research, at both academic and industrial level, combining clinical research, molecular medicine, omics-technology, and bioinformatics. When a genetics-based allocation of treatment will be a regular occurrence in oncology, PM will be successfully seen simply as "medicine".

### 2.5.1 Biomarkers for Personalized Treatment

Biomarkers are biological factors that can be quantified accurately to indicate, a specific biological mechanism, disease outcomes or therapeutic responses [12]. They can be of two types: Prognostic and Predictive and are of great importance in personalized medicine [108]. Prognostic biomarkers give information about the long-term outcome of the disease. In the clinical setting, they are mostly used for risk stratification. Predictive biomarkers are of significance before treatment, in determining which patients are likely to respond well to therapy and which are not. For example, overexpression of the biomarker *HER-2* in breast cancer patients indicates trastuzumab as the most effective treatment [108].

Establishment of advanced high-throughput technologies and Omics technology have made it easier to identify these molecular or genetic markers that can help in monitoring response to treatment. One of the most widely used method (used in this study) to identify genetic biomarkers is by analyzing genomic signatures using statistical approaches. Following this, biomarkers need to be analytically and clinically validated for their robustness, to be used efficiently in clinical practice [108].

### 2.5.2 Next Generation Sequencing

Next Generation Sequencing (NGS) represents a powerful sequencing principle based on "sequencing-by-synthesis", which means that the machine directly monitors simultaneous integration of nucleotides during sequencing reaction [109]. This technology can sequence millions of DNA templates parallelly, thereby increasing the sequencing capacity and reducing the cost remarkably. Due to the obvious benefits, NGS's increased use has enormously impacted our understanding of disease genetic

environment and is a revolutionary technology in detecting genetic variants in an individual. NGS can be applied to many DNA sources like genomic DNA (DNA), methylated DNA (epigenomic sequencing), or complementary DNA (RNA-Seq) [109]. RNA-Seq data, used in this study, provides sequencing of the entire transcriptome and reveals information about gene expressions. While NGS produces large amounts of data, the translational application of that information is only possible by analyzing the data. Thus, NGS and bioinformatic analysis go hand in hand, to make sense of the information from NGS to address a research question. NGS is a crucial tool in personalized medicine and especially beneficial in cancer, as it a genomic disease. DNA sequencing in tumor samples provides an effective strategy to capture a large amount of genomic information not only specific to that patient but also specific to the tumor tissue [110]. Genetic data produced can be used to identify suitable therapies according to the genomic signatures of individual patients. Application of NGS in a clinical setting may enormously benefit medical practice through accurate identification of disease biomarkers, detecting inherent disorders, and identifying genetic factors that can help determine response to treatment.

# 3. AIM, HYPOTHESIS AND OBJECTIVES

The research question addressed by this study is whether there exists a difference between the gene expression profiles of patients sensitive to a drug and patients resistant to a drug. The drugs under study are venetoclax and bortezomib.

**Aim:** To identify gene expression biomarkers that can be predictive of venetoclax and bortezomib drug response in Multiple Myeloma.

**Hypothesis:** The sensitivity to bortezomib and venetoclax is influenced by the expression of certain gene biomarkers. Utilizing the genomic (RNA-Seq), clinical and drug sensitivity and resistance testing (DSRT) data extracted from the MM patient samples, biomarkers associated with the venetoclax and bortezomib drug resistance can be predicted.

**Objectives:**

1. To group MM patient samples in 'resistant' and 'sensitive' for the bortezomib and venetoclax drug response using clinical and drug sensitivity and resistant testing (DSRT) data.
2. To predict differentially regulated genes in 'resistant' and 'sensitive' MM patient samples.
3. To identify the association of cytogenetic alterations with drug response to bortezomib and venetoclax.
4. To determine the biological pathways enriched in the differentially regulated genes.

The significance of this study is in its prospective application in personalized medicine. After experimentally validating the findings in larger cohorts, identified biomarkers can be used to predict suitable treatment strategies tailored for individual patients.

# 4. MATERIALS AND METHODS

## 4.1 Patient Sample Collection

Samples included in this study were collected from patients diagnosed with Multiple Myeloma. Bone marrow aspirates and peripheral blood specimens were obtained from patients during their standard treatment regiments. Patient samples were collected after informed consent and following protocols reviewed by an ethical committee of the Helsinki University Hospital (study permits 239/13/03/00/2010 and 303/13/03/01/2011) and in compliance with the Declaration of Helsinki. Patients were anonymized by the hospital. All data generated from the samples were stored in secured servers at the Institute for Molecular Medicine Finland (FIMM).

## 4.2 Drug Sensitivity and Resistance Testing (DSRT) for Venetoclax Study

### 4.2.1 Venetoclax Assay Plate Preparation

Venetoclax is a European Medicines Agency (EMA) and Food and Drug Authority (US FDA) approved oncology drug. For these studies, venetoclax was obtained from a commercial chemical vendor (ChemieTek). In compliance with the manufacturer's guidelines, venetoclax was stored in desiccators prior to being dissolved in dimethyl sulfoxide (DMSO). An Echo acoustic liquid handler from Labcyte Inc. was used to transfer the drug compound in five different dilutions (ranging from 1-10,000nM), onto 384 well plates. Storage containers pressured with nitrogen (Roylan Developments Ltd.) were used to store the assay ready plates of venetoclax to be used in the next step.

### 4.2.2 Ex vivo Drug Sensitivity Assay

Ex vivo drug sensitivity data for venetoclax were derived from earlier studies of the group [12,111]. Mononuclear cells were separated from the bone marrow aspirates by Ficoll gradient centrifugation (GE Healthcare). CD138+ plasma cells were enriched from the mononuclear cell fraction by immunomagnetic bead selection (StemCell Technologies). Plasma cells were placed in conditioned medium from the human bone marrow stromal cell line HS-5 diluted to 25% with RPMI 1640 containing 10% fetal calf serum[111] and added to the previously prepared 384 well assay plates with drugs

including venetoclax [111]. The plates were briefly shaken then incubated for about 72 hours in a controlled environment at 37° C and carbon dioxide at 5%. Cell viability was measured after 72 hours using the CellTiter-Glo assay (Promega). Dose-response curves were generated for venetoclax by feeding the luminescence intensity data derived from the plates to the software (Dotmatics Ltd.). According to the readout values, dose-response curves were fitted to quantify the venetoclax drug response on the patient samples in terms of Drug Sensitivity Score (DSS) for every sample, this was done according to previously published protocol [112]. The average difference between the DSS scores for the cells obtained from healthy individuals and patients gave rise to the Selective Drug Sensitivity Score (sDSS). High sDSS indicate higher select sensitivity of a sample towards venetoclax, while a lower sDSS corresponds to the sample being more selectively resistant to venetoclax. These results are made available to researchers on the FIMM data warehouse, *TheDB*. A datasheet was created containing the sDSS values corresponding to each patient sample for venetoclax drug molecules. These samples were also sequenced (*Section 4.4*), and the data was stored in the repository.

## 4.3 Clinical Outcome Compilation for Bortezomib Study

Treatment regimen plans and timelines of patients with multiple myeloma from the Helsinki University Hospital were compiled into clinical data. This data included clinical outcome data of 230 patients diagnosed with multiple myeloma between 1995 and 2019. The clinical data comprised of information on patient disease status, treatment, their response to the treatment, samples collected at different time points during their treatment, dosage information and blood parameters. Samples were collected from patients at different MM stages including diagnosis and relapse stages. These samples were then encoded, and results from further sequencing (*Section 4.4*) done on these samples were then stored in the database repository.

## 4.4 RNA Sequencing Analysis

Above mentioned multiple myeloma patient samples were subjected to RNA sequencing. RNA was extracted from CD138+ plasma cells using AllPrep®

DNA/RNA/miRNA Universal or miRNeasy kits (Qiagen) and then ribosomal RNA was separated from it using Ribo Zero rRNA Removal Kit. The libraries were generated by transcribing RNA to cDNA (double stranded) with the help of hexamers. RNA sequencing libraries were prepared using Scriptseq or Nextera technology and sequenced on Illumina HiSeq® 1500 or 2500 instruments (Illumina). The readout in terms of sequence reads was then filtered. Before proceeding with read mapping, the sequence reads were subjected to quality control to be filtered, trimmed, etc. These sequence reads were then aligned to the human genome reference (GRch38) using STAR aligner tool [113]. Further, the read count for each gene was revealed using an advanced software program from the subread package called featureCount[114]. *Figure 4* is a schematic representation of the process from *Section 4.1 to 4.6*.

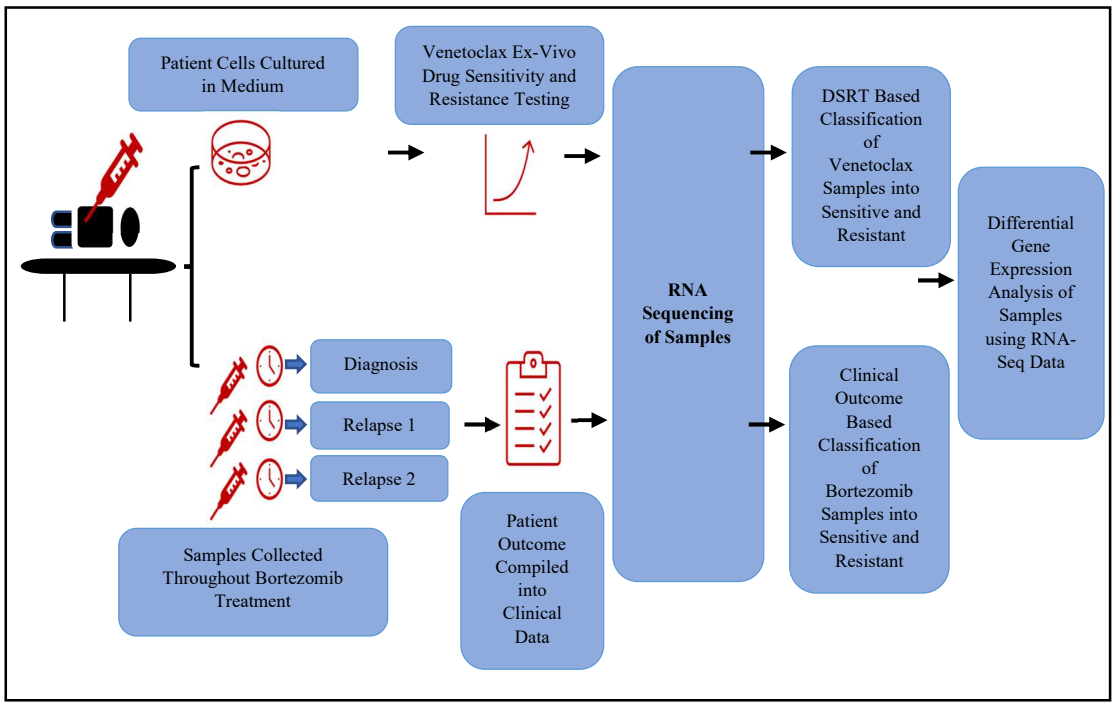


*Figure 4. Schematic representation of the study flow.*

## 4.5 Sample Classification for Data Analysis

In this study, two sub-studies are investigating the effect of the drugs venetoclax and bortezomib. The samples were selected for each study based on the availability of RNA-Seq data and classified based on other criteria specific to each study. The following is an explanation of the sample selection criteria and methodology to compile a set of data for each study containing sensitive and resistant samples.

### 4.5.1 Venetoclax Drug Study

The DSRT data containing sDSS scores of venetoclax for 200 multiple myeloma samples was obtained from the database, *TheDB*. For this study, the sample inclusion criteria were according to the selective Drug sensitivity scores (sDSS). Of the 200 samples, for only 105 samples the RNA-seq data was available, thus the rest were excluded (*Figure 5*). Based on the sDSS density distribution (*Figure 7*), samples with sDSS >17.7 (top 20% samples) were considered as sensitive and samples with sDSS < 1.97 (bottom 20% samples) were considered as resistant. With 21 resistant and 21 sensitive samples, differential gene expression analysis for venetoclax sensitivity was performed.

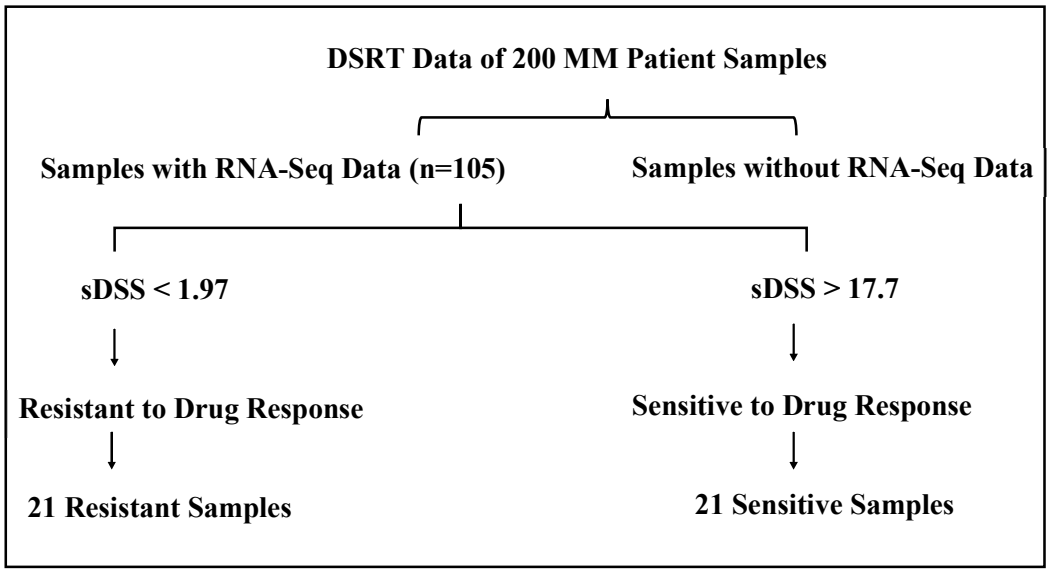


*Figure 5. Sample selection in Venetoclax study.*

### 4.5.2 Bortezomib Drug Combination Study

Among clinical data for 230 patients, 27 MM patient samples were selected for this study (*Figure 6*). These included patients' samples which, (1) had been exposed to bortezomib drug combinations, and (2) had corresponding RNA-seq data. Selected samples (n=27) were further divided into resistant (n=12) and sensitive (n=15) samples based on manual stratification using the degree of clinical response to the bortezomib drug combination treatment. Samples from patients with "Complete Remission", "Partial Remission" and "Very Good Partial Remission" consistent through multiple treatment sessions were considered as sensitive samples. On the other hand, patients with "Minimal Response", "Progressive Disease" and "Clinical Relapse" following a

few treatment sessions were considered resistant. The duration of the response degree also had an impact in deciding the category of that sample. With 12 resistant and 15 sensitive samples, differential gene expression analysis was performed to predict biomarkers for bortezomib sensitivity in multiple myeloma.

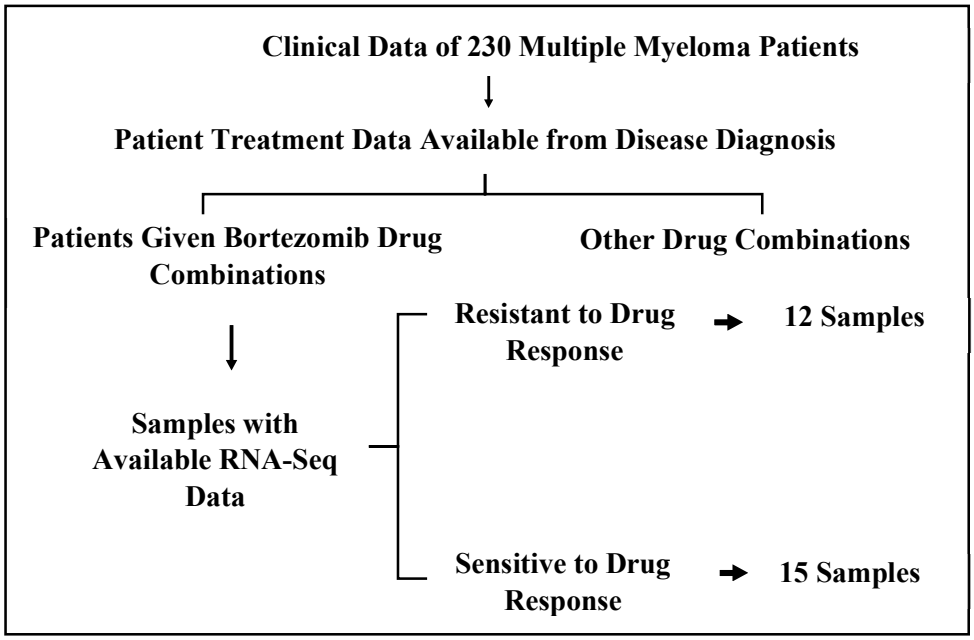


*Figure 6. Samples selection for Bortezomib study.*

## 4.6 Differential Gene Expression Analysis

Differential gene expression between the sensitive and resistant patients for bortezomib and venetoclax was identified by using the R Bioconductor[115] package, DESeq2[116]. The samples were grouped into resistant and sensitive data following which the estimate dispersion was calculated. The list of genes, with logarithmic-2-fold change values and p-values indicating the significance of differential regulation, was generated. Genes with absolute logarithmic fold change value of greater than 1.0 and p-value of $< 0.05$ were selected for further analysis.

The raw reads for all samples in each study were normalized using the Count Per Million (CPM) method where mapped reads are counts scaled by the number of sequenced fragments multiplied by one million. Normalization across samples removes biases like the sequence depths of different samples during sequencing which can prevent the read count data from accurately reflecting the differences in expression. The differential read count between the two groups (resistant and sensitive) was visualized

using heatmap.2 from the R package gplots[117]. All statistical analyses were performed using the R software environment version 3.5.2 for statistical computing and graphics.

## 4.7 Pathway Enrichment Analysis

The differently regulated genes for the venetoclax study generated by differential expression analysis were further subjected to pathway enrichment to identify their collective function, and to extract more knowledge about their involvement in response to the MM drug treatment. Pathway enrichment analysis identifies biological pathways enriched (more than by chance) in a gene list. The tool used in this study was, the Enrichr pathway analysis tool (https://amp.pharm.mssm.edu/Enrichr/)[118,119] (reference set KEGG Human 2019). The significance of the association of each pathway to the gene was given by Fisher exact test p-value (<0.05).

## 4.8 Cytogenetic Alteration Analysis

Cytogenetics data were collected from the hematology registry (Finnish Hematology Registry and Biobank). These were generated using fluorescence *in situ* hybridization technology as described previously [120], following European Myeloma Network 2012 guidelines [121]. For both venetoclax and bortezomib, cytogenic alterations were analyzed using Chi-Squared Test to indicate a significant difference between sensitive and resistant samples. The alteration landscape was visualized using heatmap.2 on R. All statistical analysis was performed using the R software environment version 3.5.2 for statistical computing and graphics.

# 5. RESULTS

## 5.1 Venetoclax Study

### 5.1.1 Sample Data Characteristics

From a group of 105 MM samples, our cohort with the most sensitive and resistant samples was selected. The sDSS values for all 105 samples ranged between -5.2 and +36.4. The bell-shaped density curve (*Figure 7*) shows the sDSS values distribution with 83.8% samples having positive sDSS values and 16.1% samples having negative sDSS values. To select the most resistant and sensitive samples and have the optimum number of samples for further analysis, 20% of all 105 samples were selected from extreme ends of the curve. This led to the formation of two sets of data containing samples that had opposite responses to venetoclax. For this study, our cohort consisted of 21 resistant samples with sDSS cutoff < +1.97 and 21 sensitive samples with sDSS > +17.7.

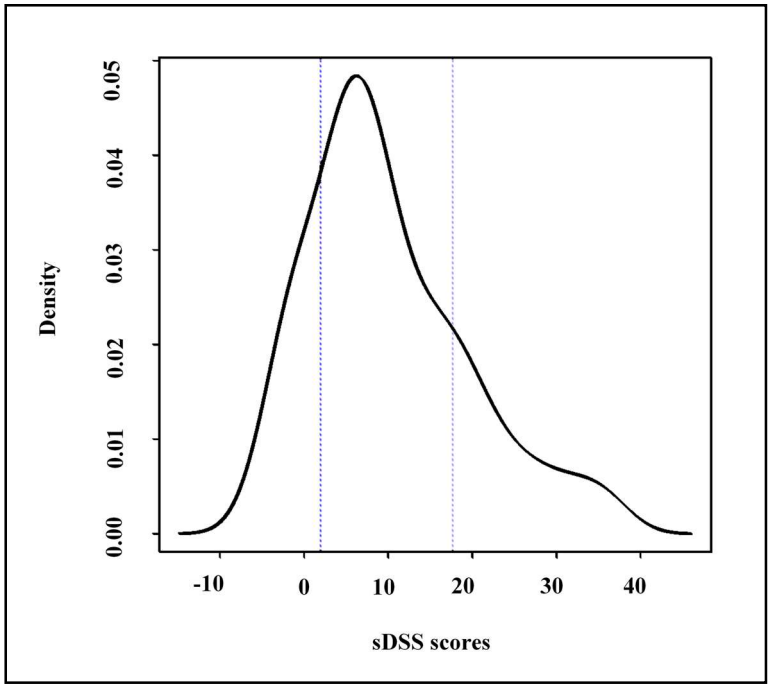


*Figure 7. sDSS values distribution curve*

### 5.1.2 Deriving Differential Gene Expression Based Profile for Venetoclax Response

We performed differential gene expression analysis between samples resistant and sensitive to venetoclax drug, as explained in *Section 4.6*. In this study, DESeq2

bioconductor R package was used to determine genes that were variably expressed between resistant and sensitive samples sets. This is one of the most widely preferred tools and analyzes based on the assumption that initially, no genes are differentially expressed. Normalization in DESeq2 is based on geometric strategy [122]. According to a comparative study conducted in 2016 [123], if replicate size is over 12 DESeq2 is the most appropriate tool for differential gene expression analysis. Following this approach, for both studies, DESeq2 was thought to be the best tool to obtain DEGs (Differentially Expressed Genes).

Differential gene expression (DGE) analysis between 21 samples most resistant to venetoclax and 21 most sensitive revealed that 152 genes differed significantly (p-value <0.05) between the two groups. These genes were identified based on an adjusted p-value of < 0.05 and fold change cutoff of 1.0. *Figure 8* shows the DGE analysis plot.

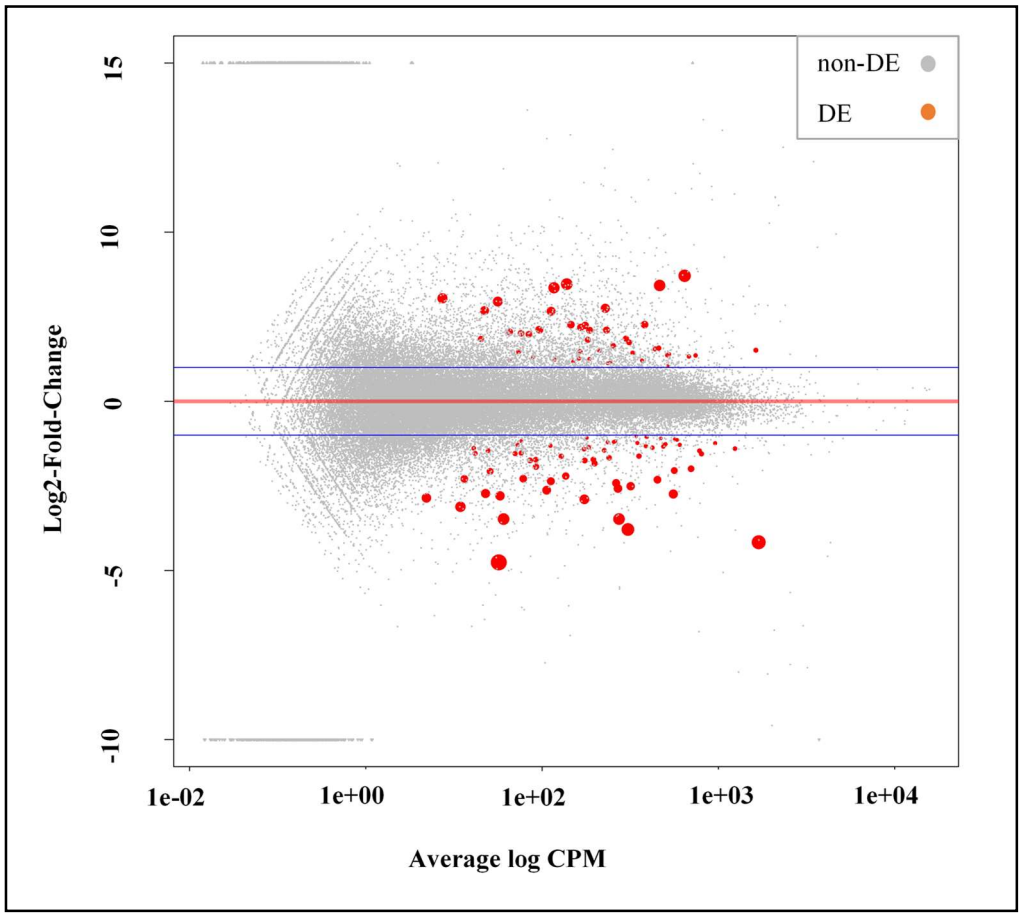


*Figure 8. DESeq2 generated differential gene expression analysis plot for venetoclax study. Total 152 differentially expressed (DE) genes identified plotted in red points. The remaining are non-DE genes plotted in gray points. Points with log2-fold> 1 change are upregulated genes and points with log2-fold change< -1 are downregulated genes in resistant samples.*

Among these, there were only 74 protein-coding genes. Genes with fold change < -1.0 (n=38) were downregulated genes (*Table 2*) in the resistant samples. Genes with fold change > 1.0 were upregulated genes (n=36) (*Table 3*) in the resistant samples. Finally, 63 protein-coding genes were selected based on absolute fold change cutoff of 1.2 and adjusted p-value < 0.05, for further use in pathway enrichment analysis.

The objective of this primary selection was to generate a list of genes that were highly variable between sensitive and resistant patient samples to further identify their significance in critical pathways of multiple myeloma. We identified a list of differentially regulated genes for the venetoclax study which were further analyzed.

*Table 2. Downregulated protein coding genes (n=38) in resistant samples generated by DESeq2 analysis for venetoclax study.*

| Gene | Log2 Fold Change | Adjusted P-value | Gene | Log2 Fold Change | Adjusted P-value |
|---|---|---|---|---|---|
| CCND1 | -4.166007883 | 0.000567022 | RNF148 | -1.447744476 | 0.028348745 |
| KCNMB2 | -3.788865208 | 0.025577166 | PDCD4 | -1.398430051 | 0.001385959 |
| KCNJ3 | -3.479514775 | 0.0216433 | SMAD7 | -1.371128024 | 0.038083608 |
| MAATS1 | -2.89686544 | 0.001385959 | ZNF711 | -1.336732626 | 0.015482515 |
| ORAOV1 | -2.74061757 | 0.025604297 | PATJ | -1.322762752 | 0.042719631 |
| RIMS1 | -2.577421649 | 0.024922454 | STAT4 | -1.29336638 | 0.00564789 |
| TSHZ2 | -2.509129876 | 0.003423159 | PIWIL2 | -1.288735773 | 0.033935206 |
| GPRASP1 | -2.416928656 | 0.042719631 | ZNF532 | -1.288146457 | 4.59E-05 |
| TRPC3 | -2.361467736 | 0.003132254 | GPR155 | -1.267416815 | 0.04348812 |
| ADAM28 | -2.046904338 | 0.004306324 | BCL2 | -1.238415417 | 0.001385959 |
| FAM107B | -1.99378328 | 0.033935206 | DOCK9 | -1.231973484 | 0.009081585 |
| STARD13 | -1.835889824 | 0.005245637 | GALM | -1.202219236 | 0.00564789 |
| TMEM220 | -1.75029327 | 0.035752465 | ZCWPW1 | -1.184187422 | 0.009575157 |
| RGPD5 | -1.725280118 | 0.046826932 | KIF21A | -1.141745254 | 0.009081585 |
| RNF133 | -1.722021288 | 0.009575157 | TET1 | -1.121641844 | 0.020009594 |
| RAPGEF3 | -1.670448423 | 0.017719008 | KIAA1147 | -1.095342711 | 0.002628055 |
| ZMAT1 | -1.620429949 | 0.005245637 | CCSER1 | -1.056855973 | 0.033935206 |
| PARM1 | -1.55386521 | 0.025604297 | HYI | -1.053289166 | 0.044348682 |
| NUGGC | -1.46299727 | 2.40E-06 | FMNL3 | -1.033589074 | 0.041003027 |

*Table 3. Upregulated protein coding genes (n=36) in resistant samples generated by DESeq2 analysis for venetoclax study.*

| Gene | Log2 Fold Change | Adjusted P-value | Gene | Log2 Fold Change | Adjusted P-value |
|---|---|---|---|---|---|
| RELN | 3.71040734 | 0.039364507 | RAB29 | 1.640650952 | 0.034795991 |
| HSD3B7 | 3.464815059 | 0.002628055 | QSOX1 | 1.571893688 | 0.027283285 |
| PXDN | 3.424730816 | 0.028348745 | ATP2B4 | 1.549551262 | 0.002628055 |
| SMIM22 | 2.948830137 | 0.039915446 | GAPDH | 1.510031272 | 0.038936859 |
| RHOU | 2.74840306 | 0.020009594 | COL4A5 | 1.506930594 | 0.039364507 |
| FJX1 | 2.681931142 | 0.003694088 | PKIG | 1.473280113 | 0.003694088 |
| PEAR1 | 2.662339377 | 0.026114061 | GLMP | 1.429459146 | 0.033935206 |
| AL645922.1 | 2.267900785 | 0.0216433 | NOMO1 | 1.385537107 | 0.028348745 |
| ARNT2 | 2.264666925 | 0.032113533 | SIAE | 1.363614486 | 0.046826932 |
| MSRB3 | 2.242113303 | 0.001453444 | MAN2B1 | 1.355672612 | 0.003809359 |
| PPFIBP1 | 2.184375091 | 0.006855673 | HIST1H2BJ | 1.324772603 | 0.046826932 |
| NEDD4 | 2.105337692 | 4.59E-05 | PTPDC1 | 1.265547538 | 0.020009594 |
| ROGDI | 2.09865185 | 0.038083608 | TMEM79 | 1.249046929 | 0.036517235 |
| PRRT1 | 2.048116424 | 0.020009594 | EXTL2 | 1.207044605 | 0.035752465 |
| HOXB7 | 2.009551707 | 0.033935206 | ZNF668 | 1.173578192 | 0.042719631 |
| SLAMF6 | 1.852896493 | 0.048014501 | FAH | 1.155517576 | 0.001385959 |
| CXADR | 1.817004758 | 0.003921473 | DHRS7B | 1.123014147 | 0.001385959 |
| KIF14 | 1.742114859 | 0.026551035 | NOMO2 | 1.043263607 | 0.025577166 |

### 5.1.3 Visualizing Variability Between Gene Expressions of Venetoclax Resistant and Sensitive Samples

To visualize the variations in gene expression patterns between sensitive and resistant samples, heatmaps were generated using *heatmap.2* from the R package *gplots*. For visualization, normalized gene expression data obtained from sequencing (*Section 4.4*) was subjected to minmax linear transformation and the data was scaled between 0 and 1. The entire range of expression values between 0 and 1 were mapped for each gene (selected after differential genes expression analysis in *Section 5.1.2*) and visualized among the resistant and sensitive groups.

The protein-coding 74 genes obtained after differential gene expression (*Section 5.1.2*) were used to map their expression across all samples. The results, shown in *Figure* 9, show a clear representation of differences between the gene expressions profiles of the two sets (resistant and sensitive).

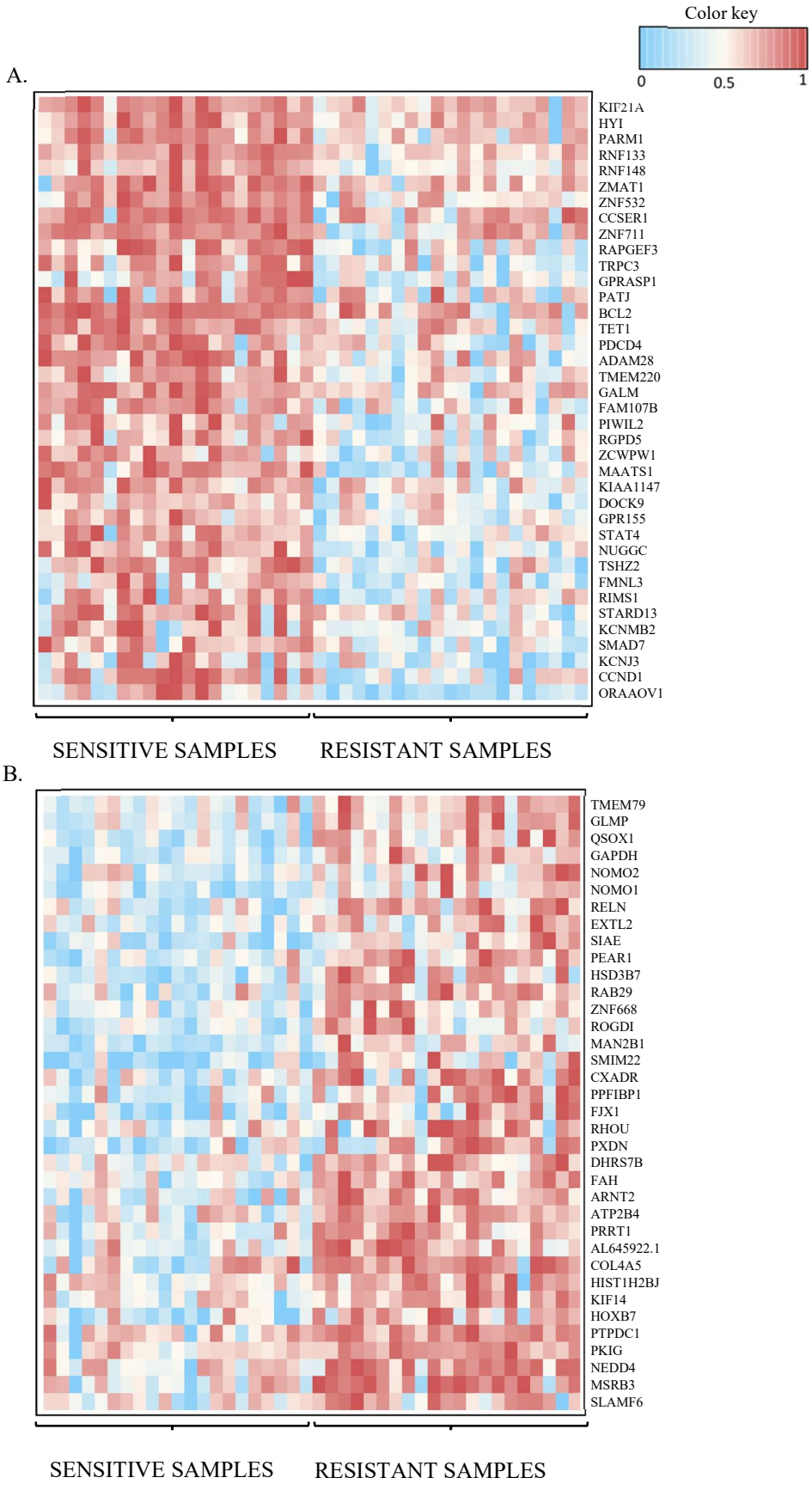


*Figure 9. Heatmap visualizing gene expression profiles of 74 DE genes across sensitive and resistant samples from venetoclax study. (A.) Heatmap of downregulated genes (n=38) obtained from DESeq analysis. (B.) Heatmap of upregulated genes (n=36) obtained from DESeq analysis. The color key indicates gene expression between 0 (lowest expression) and 1 (highest expression).*

This visualization indicates the presence of an association between varying expression profile and response to drug. Genes that are seen to be upregulated in the resistant group showed overall downregulation in the sensitive group, and vice versa. Further, expression of each of the specific 36 genes upregulated and 38 genes downregulated in venetoclax resistant samples can be distinctly observed.

Visualizing the expression profiles is an integral process that assists in cross validating the results achieved from the differential gene expression analysis. For instance, if the analysis reveals a significant gene with very high log2-fold change value but in the heatmap its expression is seen to be less consistent across replicates, then this is an indication that DGE analysis generated a false positive. Therefore, visualizing the actual gene expression is a valuable method. It is evident that for the venetoclax study, the visualized gene expression profile is consistent with the analysis.

### 5.1.4 Identifying Pathways Enriched with Differentially Expressed Genes (DEGs)

For the venetoclax study, 63 genes (top genes with absolute fold change cutoff > 1.2) resulting from the differential analysis were then further analyzed using pathway enrichment (as explained in *Section 4.7*). Fisher's exact (FE) test is used by the enrichment tool to measure the non-random association of the DEG list with the genes involved in a pathway, calculating the probability of this association. A combined score is finally calculated for each association which is the product of logarithm of FE test p-value and the z-score of deviation from the rank[118]. The higher the combined score, the better the overall ranking of the pathway and the better the p-value of the enriched gene association.

Genes with a |log2-fold change| > 1.2 and adjusted p-value <0.05, involving 31 downregulated genes and 32 upregulated genes were separately analyzed to identify the enriched pathways these were associated with. Out of 31 downregulated genes, 8 genes were most significantly (p-value <0.05) enriched in 11 pathways (*Table 4*) and among 32 upregulated genes, 4 genes were enriched most significantly in 5 pathways (*Table 5*). *CCND1, BCL2, STAT4, RELN, COL45A,* are among the top hits that critically control the pathways highlighted by the enrichment tool. The enriched pathways in the results included very crucial cancer regulatory mechanisms. The functional significance of these genes and pathways in MM resistance is further reviewed in the *Discussion.*

*Table 1. Pathways enriched with overlapping genes from downregulated DE gene list.*

| Term | P-value | Overlapping Genes |
|---|---|---|
| Hippo signaling pathway | 0.00345193048774113 | PATJ; CCND1; SMAD7 |
| JAK-STAT signaling pathway | 0.00357451390623893 | CCND1; STAT4; BCL2 |
| Hedgehog signaling pathway | 0.00360044473922809 | CCND1; BCL2 |
| p53 signaling pathway | 0.00826304538008702 | CCND1; BCL2 |
| Colorectal cancer | 0.01162064652592770 | CCND1; BCL2 |
| Small cell lung cancer | 0.01348869563408350 | CCND1; BCL2 |
| Prostate cancer | 0.01461095144404510 | CCND1; BCL2 |
| AGE-RAGE signaling pathway in diabetic complications | 0.01547831322353650 | CCND1; BCL2 |
| MicroRNAs in cancer | 0.01897485449460060 | CCND1; PDCD4; BCL2 |
| Cholinergic synapse | 0.01916225418792300 | BCL2; KCNJ3 |
| Serotonergic synapse | 0.01948440848633600 | RAPGEF3; KCNJ3 |

*Table 5. Pathways enriched with overlapping genes from upregulated DE gene list.*

| Term | P-value | Overlapping Genes |
|---|---|---|
| ECM-receptor interaction | 0.00955781492889581 | RELN; COL4A5 |
| Primary bile acid biosynthesis | 0.03017501197648500 | HSD3B7 |
| Other glycan degradation | 0.03192217639649050 | MAN2B1 |
| Focal adhesion | 0.04975267803531580 | RELN; COL4A5 |
| PI3K-Akt signaling pathway | 0.04986949005096340 | RELN; COL4A5 |

### 5.1.5 Deriving Cytogenetic Alteration Based Profile of Venetoclax Response

This study aimed to reveal that the incidence of chromosomal aberrations like translocations, deletions, chromosomal gain, etc. can significantly vary between patients sensitive or resistant to venetoclax treatment, thus it can present as a predictive factor in determining patient's response to therapy. Chromosomal aberrations, translocation at loci t(4;14), t(11;14), deletion at loci del17p13, del.13q and gain at chr1q arm were presented in a matrix, indicating the presence or absence of these

alterations across the sensitive and resistant venetoclax samples (*Figure 10)*. Visually, the profile suggests there are variations between the alterations of resistant and responsive samples, therefore Pearson's Chi-squared test with Yates' continuity correction was performed to signify the association between the cytogenetic alterations and sample response to venetoclax treatment. The relations between translocation t(11;14) and sensitive samples was significant with $X^2$ (df=1, N=21) = 12.317 and p-value = 0.0004489. Patients with t(11;14) were more likely to be responsive to venetoclax treatment. The relations between translocation t(4;14) and resistant samples was significant with $X^2$ (df=1, N=21) = 6.8234 and p-value = 0.008997. Patients with t(4;14) are more likely to be resistant to venetoclax treatment. Alterations del17p13, del.13q and gain at chr1q did not have a significant association with the treatment response.

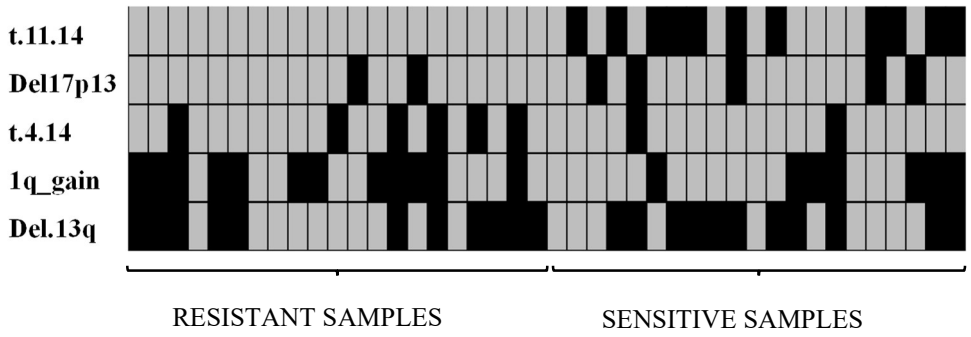


*Figure 10. Matrix visualizing cytogenetic alterations between resistant and sensitive samples from venetoclax study. Gray blocks indicate absence of alteration and black blocks indicate presence of alteration.*

## 5.2 Bortezomib Drug Combination Study

### 5.2.1 Sample Data Characteristics

Clinical data consisting of information from 230 MM patients was used to select samples sensitive and resistant to bortezomib drug combinations. Patient age at diagnosis ranged from 26-87 years with an average age of 64 years. Selection and classification criteria as explained in *Section 4.5.2* was followed to get 15 sensitive and 12 resistant samples. There were 4 distinct bortezomib drug combinations provided (*Figure 11*), (1) bortezomib, dexamethasone and lenalidomide (2) bortezomib, cyclophosphamide, dexamethasone (3) bortezomib, melphalan, prednisone (4) bortezomib and dexamethasone. Average M-protein level, one of the most significant

diagnostic parameters for MM, for sensitive samples was 38.3g/L and for resistant samples was 40.2g/L, indicating a higher M spike for samples resistant to bortezomib drug combinations.

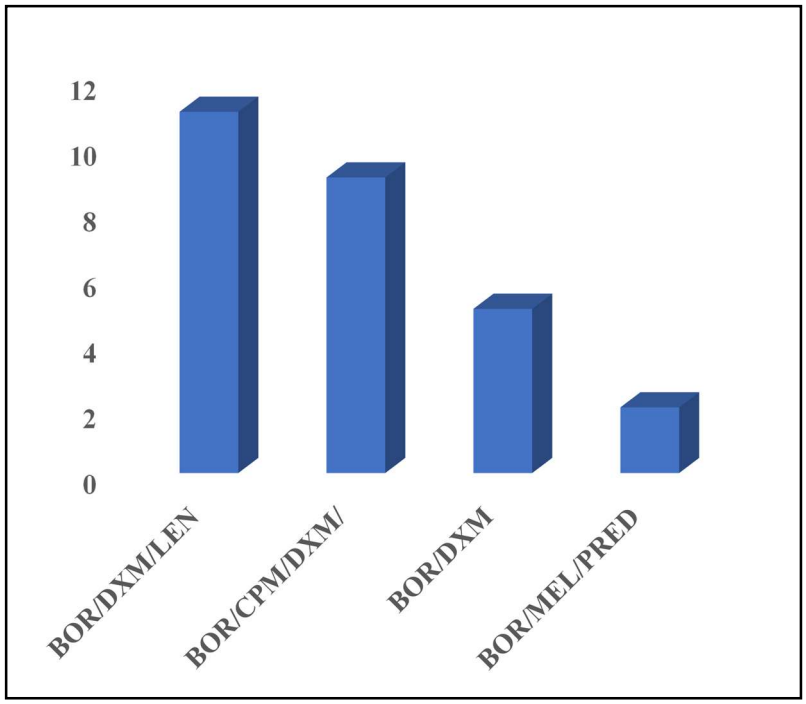


*Figure 11. Bar chart showing frequencies of different Bortezomib drug combinations given to patients. (1) BOR/DXM/LEN- Combination of Bortezomib, Dexamethasone and Lenalidomide given to 11 patients. (2) BOR/CPM/DXM- Combination of Bz, Cyclophosphamide, Dexamethasone given to 9 patients. (3) BOR/DXM- Bortezomib, Dexamethasone given to 5 patients. (4) BOR/MEL/PRED- Combination of Bortezomib, Melphalan, Prednisone given to 2 patients.*

### 5.2.2 Deriving Differential Gene Expression Based Profile for Bortezomib Response

*DESeq2* analysis for the bortezomib study identified 1 gene with significant differential gene expression between 15 highly sensitive samples and 12 highly resistant samples. *Figure 12* shows the DGE analysis plot for Bortezomib study. With adjusted p-value <0.05 and fold change of -1.09, *SVOP* gene was identified to be downregulated in the resistant samples compared to sensitive ones. Since this analysis did not reveal a conclusive list of genes, we did not proceed with further analysis for the bortezomib study. Like the cytogenetic alteration analysis done in the venetoclax study, the analysis was performed with the resistant and sensitive samples of bortezomib study; however,

it did not inform of any significant association between cytogenetic alterations and response to bortezomib drug combinations. Nonetheless, potential causes behind the unsuccessful results for this study are discussed later in *Section 6.5*.

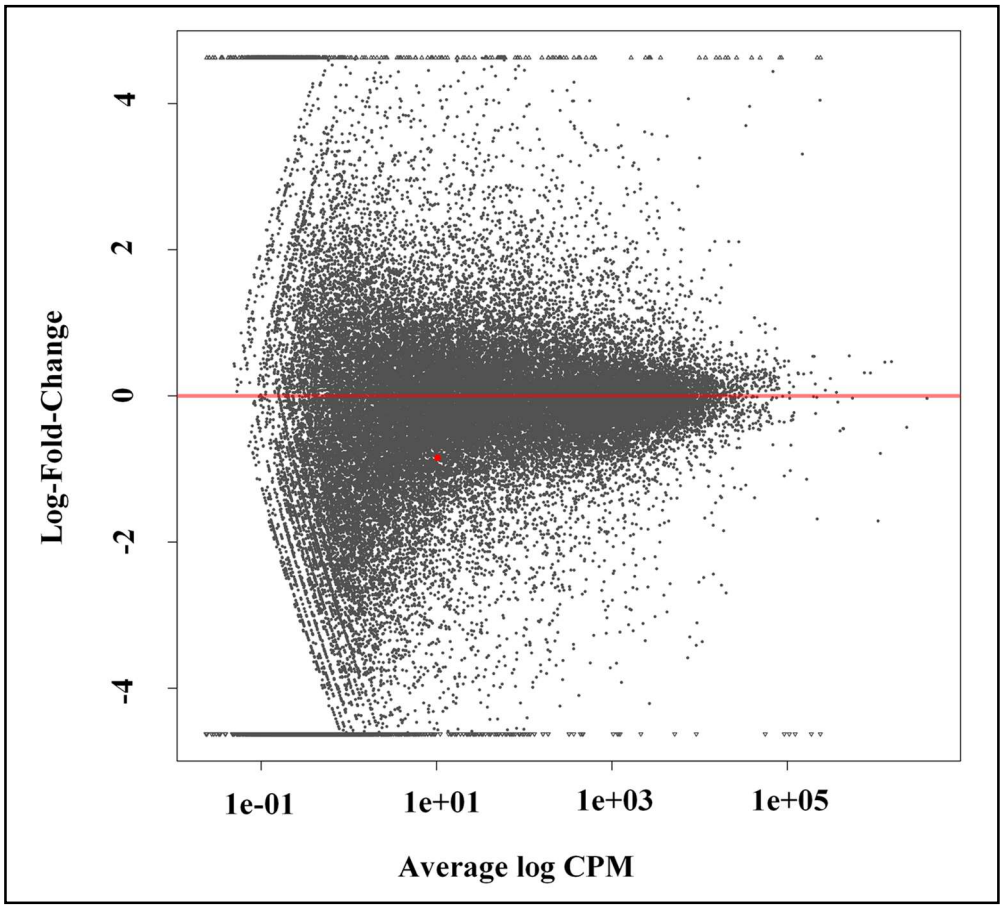


*Figure 12. DESeq2 generated differential gene expression analysis plot for Bortezomib study. Only 1 differentially expressed (DE) downregulated gene identified is plotted in red point. The remaining are non-DE genes plotted in black points.*

# 6. DISCUSSION

## 6.1 Differential Gene Expression Profiling: A Translational Approach

We used RNA-Seq data to investigate differential gene expression patterns of many genes between sensitive and resistant samples and identified predictive biomarkers. A multitude of studies have used gene signature profiling to identify prognostic markers for multiple myeloma, but there are not many where gene signatures are analyzed in response to specific drug treatments. Furthermore, most of the differential gene expression-based studies are done on human myeloma cell lines that do not always reflect the accurate genetic and phenotypic make-up of their tissue of origin nor the heterogeneity of the disease. Whereas using CD138+ plasma cells obtained from patient samples for RNA-Seq and ex vivo drug response provides precise information regarding the important markers and functions. The bortezomib study incorporates clinical profile-based drug response, which is the best representation of in vivo microenvironment changes.

We successfully identified venetoclax-associated biomarkers to create a gene panel that can be further explored and validated. Many genes identified in our study have been previously associated with either disease progression or prognosis in multiple myeloma. By using systemic tools to explore large scale genomic data, this study is a translational approach to predicting drug response biomarkers and has the potential to translate into the clinical setting.

## 6.2 Varying Gene Expressions Can Drive Venetoclax Resistance in MM

The differentially regulated genes identified for venetoclax response play crucial roles in cancer pathways that might impart resistance to drug action. In this section, a literature-based previously discovered association of the genomic signatures with MM resistance will be discussed, with respect to the gene list discovered in our study.

Reelin protein translated from gene *RELN* is an extracellular matrix (ECM) glycoprotein responsible for ECM-receptor interactions of neuronal cells. Overexpression of *RELN* is associated with poor progression-free survival in patients

with multiple myeloma [124]. In 2016, a study investigated the expression of *RELN* in MM and it was found that it facilitates the adhesion of malignant cells to fibronectin thereby fostering resistance against drug-induced cell apoptosis [125]. In our study, *RELN* was the most significantly upregulated gene in the venetoclax resistant cohort (*Table 3; log2-fold change :3.7; p-value: 0.03*) and was also found enriched in ECM-receptor interaction and focal adhesion pathways (*Table 5*).

MM spike genes are genes that are overexpressed in myeloma cells. A study conducted in 2011, identified an association between these genes and poor MM prognosis with decreased treatment sensitivity [126]. *RHOU, MSRB3, HIST1H2B, HOXB7* and *COL4A5* were some of the spike genes identified with high bad prognostic values. Remarkably, these genes are significantly overexpressed in our venetoclax resistant samples (*Table 3*).

Another study in 2013, identified the association of overexpressed *KIF14* along with *ABCB1* in imparting resistance against MM therapy in cell lines that were positive for t(4;14) [127]. Our study reveals significant upregulation of *KIF14* in resistant samples (*Table 3; log2-fold change: 1.74; p-value: 0.026*) that also are significantly positive for t(4;14) translocation (*Figure 10*).

STAT family of proteins play a very crucial role in regulating cellular proliferation, differentiation, and apoptosis. Role of *STAT3* in driving cancer metastasis is a very well-investigated phenomenon, though, interestingly, it did not show up in the DEG profile. Instead, our study shows a significant downregulation of *STAT4* in resistant samples (*Table 2; log2-fold change: -1.29; p-value: 0.005*). The exact function of *STAT4* in cancer progression is limited and not yet investigated deeply, however, some studies have shown the association of high *STAT4* expression with good prognosis and treatment sensitive in gastric cancers [128], ovarian cancers [129] and liver cancers [130]. It could be inferred that *STAT4* associates with good prognosis and sensitive drug response in myeloma.

Drug resistance in multiple myeloma has been associated with lower expression of the transient receptor potential channel (TRPC) family of genes [131]. Thus, downregulation of *TRPC3 (Table 2; log2-fold change: -2.36; p-value: 0.003)* in resistant samples is a potential indicator of venetoclax response in MM.

The *BCL2* gene is responsible for producing proteins that facilitate the inhibition of cellular apoptosis, thus are found to be upregulated in hematologic malignancies. Since venetoclax is a BCL2 specific inhibitor, one might speculate that a lower expression of *BCL2* in patient will lead to a positive response to venetoclax. However, a recent study demonstrated that *BCL2* expression effects drug response in different ways for different hematological malignancies [132]. For multiple myeloma, lower expression of *BCL2*, *BCL2L11* and *BAX* along with overexpression of *BCL2L1* gives rise to a bad Overall Response Rate (ORR) to venetoclax and poor Progression Free Survival (PFS) as compared to CLL (for which venetoclax is the first line of treatment, approved in the United States) [132]. In this study, *BCL2* downregulation was identified in resistant venetoclax samples (*Table 2; log2-fold change: -1.23; p-value: 0.001*), therefore a lower expression of *BCL2* in multiple myeloma patients could be predictive of poor venetoclax response.

## 6.3 Identified Pathways are Associated with Drug Resistance in MM

In pathway enrichment analysis, several important pathways were enriched with genes like *BCL2*, *CCND1*, *SMAD7*, etc. (*Table 4 and Table 5*). Deregulation of the genes involved in these pathways may lead to acquired resistance against treatment.

The bone marrow microenvironment can impart resistance to myeloma therapy mainly through two mechanisms, (1) cell adhesion mediated interaction of the malignant cells with fibronectin and (2) inhibition of apoptosis due to cytokine driven actions, which involve both JAK/STAT pathway and PI3K/AKT signaling [133]. In our study, important genes *RELN* and *COL2A4* were found upregulated in resistant samples. These genes were enriched in pathways (*Table 5*), ECM-receptor interaction, focal adhesion and PI3K/AKT signaling, which are involved in supporting survival of malignant myeloma cells [133], thereby leading to progression of resistant disease.

Hedgehog pathway and genetic factors involved in this pathway play a major role in modulating the stem cell environment in MM [134]. Abnormal activation of hedgehog signaling is promoted by deregulation of factors like BCL2 and causes clonal expansion in MM [133]. Results from our study are consistent with the involvement of hedgehog pathway in MM disease progression (*Table 4*).

Pathway enrichment in this study identified p53 pathway enriched with downregulated genes in resistant samples (*Table 4*). Attenuation of p53 pathway is associated with tumorigenesis and confers resistance to treatment not in MM but many cancers [135]. Hippo signaling (*Table 4*) plays a crucial part in apoptosis of lymphocytes and are therefore very important in MM progression [136]. Their downregulation is associated with aggressive cancer development and signaling is linked with TGFβ pathway involving the SMAD family of proteins [136136].

## 6.4 *CCND1* Expression and Chromosomal Translocations Predict Response to Venetoclax in MM

The *CCND1* gene expresses Cyclin D1, a protein that causes the release of E2F, a transcription factor, through phosphorylation of retinoblastoma [137]. This process leads to the transition of cell cycle from G1 to synthesis phase resulting in cellular proliferation and DNA replication. Deregulation of *CCND1* is usually the case in most malignancies, but it is most common in Multiple Myeloma [137]. Overexpression of *CCND1* gene occurs due to a translocation alteration t(11;14) [63]. Patients with t(11;14) have high levels of Cyclin D protein. t(11;14) and *CCND1* overexpression has been shown to predict venetoclax drug response in multiple myeloma [138]. Translocation t(11;14) is one of the most common alterations in myeloma patients and occurs early in disease development causing the upregulation of *CCND1*. Previous studies have demonstrated that presence of t(11;14) and overexpressed *CCND1* has been associated with positive drug response in myeloma [12] and in our study, venetoclax resistance does correspond with downregulated *CCND1* expression (*Table 2; log2-fold change: -4.16; p-value: 0.0005*). Moreover, patients with t(11;14) were more likely to be responsive to venetoclax treatment (*p-value = 0.0004*) in our sensitive cohort.

Translocation t(4;14) occurs in about 10%-25% of myeloma patients and translocation of genes into this location leads to their overexpression [139]. It is usually associated with overexpression of *FGRF3* and *MMSET* domain that promotes myeloma disease progression. t(4;14) is known to be associated with resistance against venetoclax [12]**Error! Bookmark not defined.**. In our study, resistant samples have a significantly higher number of t(4;14) as compared to sensitive samples (*p-value: 0.008; Figure 10*).

Therefore, our study identified t(4;14) as a biomarker for resistance to venetoclax in MM and *CCND1* and t(11;14) as indicators of sensitivity.

## 6.5 Limitations of Bortezomib Drug Combination Study

Only 1 differentially regulated gene was obtained for the bortezomib study and the results from the cytogenetic analysis were not conclusive, unlike the venetoclax study. One reason for this could be the small cohort size (n=27) of the bortezomib study as compared to the venetoclax study (n=42). The samples were characterized (into resistant and sensitive) based on the clinical response of patients to bortezomib. However, during treatment administration, patients were given 4 different drugs in combination with bortezomib (refer to *Section 5.2.1*) instead of just solely being administered with bortezomib (as was the case with the venetoclax study). Moreover, patients also received other medications for controlling side effects or for some other conditions that were not considered in this study. When there are so many pharmacological factors involved, cascades of changes happen in the microenvironment which ultimately affects the gene expression patterns. This could be a reason for the poor result from differential gene expression analysis of samples from patients administered with bortezomib drug combinations.

In order to get more clarity on this, we did a small graphical correlation between clinical response-based profiling of patients (into resistant and sensitive) administered with bortezomib drug combination and sDSS bortezomib score-based profiling of samples from the same patients. *Figure 13* shows that samples deemed resistant based on clinical outcome of bortezomib drug combinations were not resistant based on the sDSS scores of bortezomib ex vivo study.

This shows a lack of correlation between clinical and ex vivo studies for bortezomib. While further exploration into this was out of the scope of this study, investigating the differences between clinical and ex vivo studies would be a valuable experiment which can improve the application of ex vivo drug testing in the clinical setting.

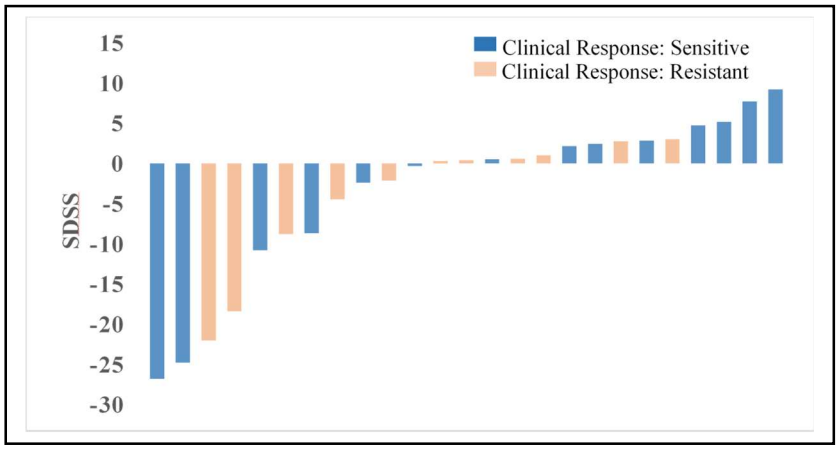


*Figure 13. A bar chart showing the correlation between clinical response-based profiling of patients (into resistant and sensitive) and sDSS score-based profiling of samples from the same patients. Positive sDSS value indicates sensitive response and negative sDSS indicates resistant response.*

## 6.6 Conclusion and Future Prospects

Despite the development of therapeutic strategies, multiple myeloma remains an incurable disease with a standing issue of ultimately relapsing in initially responsive patients. This study aimed to tackle that problem by developing a methodology to predict patient response to drugs. In this study we identified 74 protein-coding gene biomarkers of venetoclax resistance in MM, and 2 cytogenetic alterations associated with venetoclax resistance. This demonstrates that comparative study of gene expression signatures and cytogenetic alterations can indicate patient response to a specific treatment. Our primary goal was to identify predictive biomarkers for drug response; however, this is only an elementary step that needs further validation and verification. The over/under-expression of the identified genes can be verified by quantitative PCR analysis of the patient samples. Further investigation into exploring the pathways through which the identified genes function, will give: (1) insight into resistance acquiring mechanisms and (2) novel drug targets for resistant multiple myeloma. A similar analysis could be done for a larger sample size and for a wider range of data (like proteomics data), which would enhance the translational impact in clinical settings. The results from this study provide a basis for the development of further research that is guided by patient's genomic landscape, verified in large cohorts, applicable in the clinical setting and results in personalized targeted treatment strategies. This study is a small contribution towards realizing the goal of personalized medicine eventually becoming the norm.

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